Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center

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Executive Summary

The goal of the experiments described in this report was to evaluate the toxicity of fine particulate matter (PM) derived from the destruction of the World Trade Center (WTC) on the respiratory tract of mice, and thereby contribute to the short-term health risk assessment of WTC PM being conducted by the Environmental Protection Agency. The adopted approach allowed a comparison of the intrinsic acute toxicity of fine WTC PM in the respiratory tract to well-studied PM reference samples that range in toxicity from essentially inert to quite toxic. The fundamental question was whether fine WTC PM was uniquely highly toxic.

This toxicological research complements efforts by EPA and other organizations to assess the extent and level of worker and public exposures to PM derived from the WTC disaster and recovery efforts. This research is informative, but it is of limited scope, with a focus on the toxicological effects of the fine fraction of WTC dust from a single exposure. A more complete characterization of potential health effects would include consideration of other size fractions, repeated exposures, additional doses and endpoints, and responses in species or strains of differing sensitivity. It was not possible to assess these other considerations in the present study.

Fallen dust samples were collected on September 12 and 13 from various sites around Ground Zero, and the fine PM fraction (< 2.5 microns in diameter; $PM_{2.5}$) was isolated on filters. $PM_{2.5}$ was extracted from the filters and extensively analyzed by several chemical and physical techniques. A dose-response study in mice was conducted comparing aspirated WTC $PM_{2.5}$ (pooled from 7 different locations near the WTC site) with low and high toxicity $PM_{2.5}$ control samples (Mt. St. Helens and residual oil fly ash (ROFA), respectively). An acute nose-only inhalation exposure study was conducted on one WTC $PM_{2.5}$ sample, since upper airway irritation has been a primary complaint of those living and working in the WTC area. Finally, a short-term time course study was conducted comparing aspirated samples from the 7 different locations with each other and with a standard $PM_{2.5}$ sample (NIST 1649a, an ambient air PM sample collected in Washington, DC).

Fine size-fractionated WTC $PM_{2.5}$ was composed primarily of calcium-based compounds such as calcium sulfate (gypsum) and calcium carbonate (calcite, the main component of limestone). These and other compounds and elements found in the WTC $PM_{2.5}$ samples are indicative of crushed building materials such as cement, concrete aggregate, ceiling tiles, and wallboard. Levels of carbon were relatively low, suggesting that combustion-derived particles did not form a significant fraction of these samples recovered in the immediate aftermath of the destruction of the towers. Gypsum and calcite are known to cause irritation of the mucus membranes of the eyes and respiratory tract.

Samples of WTC $PM_{2.5}$ induced mild to moderate degrees of inflammation when administered at a relatively high dose (100 μg) directly into the airways of mice. The pulmonary inflammatory response was not as great as that caused by the reference $PM_{2.5}$ samples (toxic ROFA and ambient air NIST 1649a). However, this same dose of WTC $PM_{2.5}$ caused airway hyperresponsiveness (a

greater sensitivity to agents which constrict breathing passages) comparable to NIST 1649a and to a greater degree than ROFA. Doses of 10 and 32 μg administered directly into the airways, or inhalation at 10 mg/m^3, did not induce significant inflammation or hyperresponsiveness. The significant degree of airway hyperresponsiveness induced by the high dose of WTC $PM_{2.5}$ implies that components of the dust can promote mechanisms of airway obstruction.

The results from these studies indicate that a high dose of WTC dust as $PM_{2.5}$ would be necessary to elicit effects in healthy people. Hypothetical calculations are presented indicating that a healthy worker at Ground Zero would have to inhale about 425 μg/m^3 WTC $PM_{2.5}$ for 8 hours to achieve the same dose per tracheobronchial surface area as occurred with the high dose of WTC $PM_{2.5}$ used in the mouse studies. These high concentrations are conceivable in the aftermath of the collapse of the towers when rescue and salvage efforts were in effect. Therefore a healthy worker without respiratory protection could have inhaled enough WTC $PM_{2.5}$ to cause pulmonary inflammation, airway hyperresponsiveness, and manifestations of sensory irritation such as cough. Species differences in responses to inhalation of WTC $PM_{2.5}$ are unknown and were not considered in these calculations. Individuals who are especially sensitive to inhalation of dusts, such as asthmatics, may experience these effects at lower doses of inhaled WTC $PM_{2.5}$. These studies suggest that most healthy people would not respond to a single exposure to moderately high WTC $PM_{2.5}$ levels (about 130 μg/m^3 or less for 8 hours) with any adverse respiratory responses. However, it should be emphasized that the effects of chronic (long-term) or repeated exposures to lower levels of WTC $PM_{2.5}$, or the persistence of any respiratory effects are unknown and were not components of this study. Although only fine $PM_{2.5}$ was tested in these experiments, its composition was similar to coarser PM, suggesting that biological responses to both size fractions within the respiratory system may be similar. The results of these studies will need to be placed within the context of an overall risk assessment for exposures to pollutants generated by the World Trade Center disaster.

I. Introduction

The World Trade Center (WTC) disaster sparked enormous concern about the quality of the environment in the surrounding neighborhoods. One of the immediate concerns was the effect of dust from the collapse and burning of the towers on breathing, especially in more susceptible individuals. Dust infiltrated indoors into homes and apartments, in many cases up to several inches in depth. Fires at the WTC site continued for several months before finally being extinguished, and emitted significant quantities of particulate matter (PM). Recovery and reconstruction efforts have also contributed to emissions of fine (< 2.5 microns; $PM_{2.5}$), coarse (> 2.5 and < 10 microns; $PM_{2.5-10}$), and larger (> 10 microns) size PM fractions. The dust particles from the WTC site appear to be quite alkaline in nature, probably due to partial dissolution of concrete, gypsum, and glass fiber particles (USGS, 2002). As people are trying to move back, decisions must be made about cleaning procedures since potential exposure issues are associated with redispersal and residual dust.

Those moving back to their homes as well as those who work in the area have reported throat irritation, cough, and other indications of mucous tissue sensory irritation (New York Times, 2001; Washington Post, 2002). Nose and throat irritation may be caused by particles which deposit in the nasal passages and upper airways and stimulate sensory nerve reflexes (Costa and Schelegle, 1999). Airborne dust may elicit inflammation, mucus production, coughing, and sneezing in an effort to clear the lung of particles (Raabe, 1999). However, inflammation, mucus production, and airway hyperresponsiveness may all contribute to airway obstruction. Since asthma is characterized by all of these cardinal features (Sears, 1997), it is logical to suspect that asthmatic individuals may be more sensitive to agents which further promote airway obstruction.

The National Exposure Research Laboratory (NERL, USEPA), in coordination with Region 2 of the U.S. Environmental Protection Agency (USEPA or EPA) and the New York Department of Environmental Protection (NYDEP), has been monitoring ambient pollutants including volatile organic compounds (VOCs), dioxins, and PM in an effort to ascertain exposures. In addition, New York University (NYU) and Rutgers University have collected bulk samples of ash and dust in the immediate aftermath of the disaster. The National Health and Environmental Effects Research Laboratory (NHEERL, USEPA) has collaborated with these organizations to study health effects of PM from the immediate vicinity of the WTC site.

The primary goal of the present study was to evaluate the potential health effects of PM in people working or living in the vicinity of the WTC and downwind of fires and dispersed building materials immediately after the WTC collapse. Toxicologic assessment of entrained (settled) dusts and combustion-derived PM dispersed in the areas surrounding the WTC will provide basic hazard identification information from which a broad health assessment may be derived. These findings would provide objective information to EPA, New York State, and local authorities to communicate to the public about collateral public health concerns.

In order to begin assessment of the toxicity of dust derived from the destruction of the WTC towers, scientists from NYU (led by Drs. Lung Chi Chen and Mitchell Cohen) went to the area around "Ground Zero" on September 12 and September 13, 2001. They collected bulk samples of settled dust from several sites in the immediate vicinity (<0.5 miles). Back in their laboratories at NYU, they utilized a procedure to size-fractionate the dust to obtain both fine and coarse PM fractions which can be readily inhaled and deposit in the respiratory tract, and are therefore relevant for study of toxicological effects. On October 2, 2001, Dr. Chen contacted Dr. Daniel L. Costa of the U.S. EPA NHEERL in order to collaborate on investigations of the toxicity of these size-fractionated WTC PM samples.

The approach of the present study (code name WTC2001) was to compare the toxicity of samples of size-fractionated WTC $PM_{2.5}$ with previously tested PM samples in mice. Mice offer a number of advantages for toxicity studies: 1) less sample is needed to assess toxicity;

2) the biology of the mouse has been intensively studied in the scientific literature; 3) a wide array of mouse-specific analytical reagents is available; and 4) we have extensive experience in assessing physiological responses, inflammation, and respiratory tract injury in mice exposed to other samples of air pollutants. The WTC $PM_{2.5}$ samples were thoroughly characterized by a number of chemical and physical techniques in order to compare the composition of the samples with other reference samples. A dose-response study in mice was conducted comparing aspirated WTC $PM_{2.5}$ (pooled from 7 different locations near the WTC site) with low and high toxicity $PM_{2.5}$ control samples. An acute inhalation exposure study was conducted on one WTC $PM_{2.5}$ sample, since upper airways irritation is a primary complaint of those living and working in the WTC area. Finally, a short-term time course study was conducted comparing aspirated samples from 7 different locations with each other and with a standard $PM_{2.5}$ sample.

Several methods were common to all three of these experiments to determine the toxicological effects of WTC $PM_{2.5}$. The ability of these $PM_{2.5}$ samples to affect respiratory tract responsiveness to aerosolized methacholine was determined. Since this chemical triggers airway narrowing, the test is appropriate to determine sensitivity to agents which induce airway obstruction. Bronchoalveolar lavage is a common standard technique which quantifies numbers of inflammatory cells and levels of proteins and enzymes indicative of lung injury. Lung pathological effects were assessed in a semi-quantitative fashion in all studies, and pathological effects in the nasal region were determined in the inhalation study. Comparison of the toxicological effects of dust derived from the destruction of the WTC with $PM_{2.5}$ samples which have been extensively characterized in the literature will be clearly beneficial and relevant to the overall assessment of health consequences of environmental pollutants related to this disaster.

II. Materials and Methods

A. WTC PM Sample Collection and Size Fractionation

On 9/12/2001 and 9/13/2001, scientists from New York University went to the WTC area to collect bulk samples of fallen dust. Using a paper scoop, bulk samples were taken from various outdoor locations (e.g. car hood, window ledge, park bench) as well as one indoor location, all of which appeared undisturbed since the collapse of the towers, as judged by the presence of a smooth uniform layer of dust and the absence of indicators of recent human activity. Thirteen samples were collected and labeled with numbers (1 - 13) on 9/12/2001, and six samples were collected and labeled with letters (A - F) on 9/13/2001. Samples were stored in 75 ml or 250 ml polystyrene flasks at room temperature. All samples were collected before rain fell on 9/14/2001, which certainly altered chemical and physical characteristics of the dust. Samples were taken back to NYU for processing to isolate different size fractions.

Bulk samples of dust were sieved with a 53 μ mesh screen (USA Standard Testing Sieves, Fisher Scientific, Pittsburgh, PA) on a shaker (Portable Sieve Shaker, Tyler Industrial Products, Mentor, OH). The sieved material (PM_{53}) was aerosolized through a 10 μ cut inlet to remove particles in the 10 - 53 μ range and isolate the PM_{10} fraction. The PM_{10} fraction then passed through a 2.5 μ cyclone (made in house) to remove the $PM_{2.5-10}$ (coarse) fraction and isolate the $PM_{2.5}$ (fine) fraction. The $PM_{2.5}$ fraction was collected on Teflon filters (Pall Gelman Sciences, Port Washington, NY - Zefluor Supported PTFE, 2 micron pore size, 47 mm, part # P5PJ047). While fractionating the PM samples, the filters became loaded and slowed airflow. Consequently, loaded filters were replaced with fresh filters periodically, and about 10 – 40 filters were used to completely size-fractionate each WTC sample. Analysis of the weights found in the 4 size fractions showed that roughly half of the sample was in the PM_{53} sieved fraction. Of the PM_{53} fraction, about 80-89% was in the 10 - 53 μ size range, which is too large to use in respiratory toxicology studies since only 45% of 10 μ particles are even inhalable in small laboratory animals (Menache et al., 1995), and deposition of particles greater than 5 μm is minimal (Raabe et al., 1988). The amount of the 2.5 - 10 μ fraction was very small (0.04 - 1.14 % of the PM_{53} fraction, except 3.23% in sample 13) and was therefore not feasible to study. The $PM_{2.5}$ fraction, however, was present in large enough amounts (2.29 - 4.06 % of PM_{53} fraction) to study for potential respiratory health effects, and is toxicologically relevant since it is associated with epidemiological findings of health effects in humans (Dockery et al., 1993). [The sum of the size fraction percentages does not total 100% of the original PM_{53} fraction because of loss of sample during fractionation steps.] After examination of the available inventory of filters and the locations where the samples

Figure 1. WTC dust samples were collected by New York University (NYU) scientists from 13 sites on 9/12/2001 (numbers) and from 6 sites on 9/13/2001 (letters). Collection sites are shown only for samples used in the WTC2001 study. Map provided by MapQuest.com, Inc.

Figure 2. WTC bulk dust samples were size-fractionated by NYU. Filters containing the $PM_{2.5}$ fraction were received at the U.S. EPA in Research Triangle Park, NC on 10/26/2001, and were inspected and photographed 10/29/2001.

were collected, filters containing the $PM_{2.5}$ fraction were selected from seven locations (sites 8, 11, 13, B, C, E, and F) around Ground Zero, in order to assess toxicity of samples from different geographical locations as well as overall toxicity of a pooled sample from these locations (Figure 1). The locations were selected to represent a distribution surrounding the WTC site, with more collection sites in the east reflecting the predominant winds in that direction.

Fourteen Teflon filters containing the $PM_{2.5}$ fraction from the 7 different sites collected around the World Trade Center on 9/12/01 and 9/13/01 were shipped by overnight express to EPA and received on October 26, 2001, and these were inspected and photographed on October 29, 2001 (Figure 2). [Throughout the WTC2001 study, sample transfers were accompanied by signed chain-of-custody letters]. A total quantity of about 50 mg from each site, collected on 1 to 3 filters per site, was provided. The weight of $PM_{2.5}$ on the filters was determined by NYU, and was separately determined at EPA after overnight dessication using a Cahn electrobalance. The description of the locations of the 7 samples and the total weight of $PM_{2.5}$ on the filters from each site is provided in Table 1. $PM_{2.5}$ could not be efficiently scraped off of one filter, so it was necessary to isolate the $PM_{2.5}$ using an aqueous extraction procedure (see below).

Throat irritation, cough, nosebleeds, and other mucous tissue/sensory irritation were reported by residents and workers in the WTC area (Washington Post, 2002). Oropharyngeal aspiration of PM bypasses the nose and therefore potentially relevant effects may go undetected. Consequently, it was decided that an inhalation exposure study should be conducted which might reveal important information about the toxicity and mode of action of WTC $PM_{2.5}$. Since there was not enough $PM_{2.5}$ or $PM_{2.5\text{-}10}$ sample available to conduct an inhalation exposure study (> 2 g necessary), it was decided to use a PM_{53} sample (sieved but not further fractionated) which was available in large enough quantities to run through the inhalation exposure system. The EPA inhalation exposure system has a 2.5 μm cut–point cyclone to remove larger particles (Ledbetter et al., 1998), and therefore measurement of the PM concentration in the exposure zone of the chamber represents exposure to $PM_{2.5}$. A sample of PM_{53} from location #3 (figure 1), 0.3 miles east of Ground Zero (in the predominant wind direction), was available in large enough quantities for the nose-only inhalation exposure study. This sample was sent by overnight express from NYU and received on November 21, 2001.

B. Extraction of PM from Teflon Filters

Filters were extracted using a modification of a method by Biran and coworkers (1996). Each filter was handled with clean sterilized stainless steel forceps. Filters from each of the 7 individual collection sites (1 - 3 filters per site) were extracted into a single volume of distilled water (Gibco BRL ultrapure 10977-015, lot 1063705) in the ratio of 0.5 ml water per mg sample (2 mg PM / ml water; range 24.96 - 27.14 ml). This volume of water was pipetted into a 100 ml sterile plastic specimen cup containing a 3 mm thick Teflon ring at the bottom of the cup designed to support the filter. Filters were wetted with 200 μl of 70% ethanol on the particle side. The liquid was gently spread on the filter with the pipet tip, taking care not to scrape the filter. The filter was then placed on top of the 3 mm thick Teflon ring in the specimen cup with the particle side down, and a 6 mm thick Teflon ring was placed on top of the filter. The cup with the filter was secured to an orbital shaker (Titer Plate Shaker, Lab-Line Instruments, Melrose Park, IL). A cleaned sonicator probe (18 mm diameter, Sonic 300 Dismembrator, Artek Systems Corp., Farmingdale, NY) was rinsed with 1% Triton X-100 (Sigma Chemical Co., St. Louis MO; T8787), and then ultrapure distilled water (Gibco BRL ultrapure 10977-015, lot 1063705) before and after each extraction. The probe was then lowered into the water in the specimen cup to a level just above the filter. Ice was placed around the specimen cup to prevent rising temperatures during sonication, and the temperature of the water was measured before and after sonication. The shaker was turned to the lowest speed at which it would run continuously (setting = 2). The sonicator power was set to 30, and the filter was sonicated for 30 minutes while rotating on the orbital shaker. After sonication, the filter

Table 1. Description of Samples Used in WTC2001 Study

WTC Site Samples

Experiment	Sample Code	Collection Date	Location, Description	Size Fraction	Total Weight on Filters	Extracted Wt., % Extracted
A, C	WTC 8	9/12/2001	Beekman Street - filters # 9, #14 0.4 miles E of Ground 0 center	$PM_{2.5}$	53.316 mg	46.70 mg 87.6%
A, C	WTC 11	9/12/2001	55 Church Street - filters #13, #14, #15 In front of Millenium Hilton Hotel 0.1 miles E of Ground 0 center	$PM_{2.5}$	50.097 mg	29.79 mg 59.5%
A, C	WTC 13	9/12/2001	Church & Liberty St. - filters #4, #5 0.1 miles SE of Ground 0 center	$PM_{2.5}$	51.006 mg	46.29 mg 90.8%
A, C	WTC B	9/13/2001	Trinity & Rector - filter #4 From a car hood and windshield 0.25 miles S of Ground 0 center	$PM_{2.5}$	52.969 mg	42.31 mg 79.9%
A, C	WTC C	9/13/2001	Winter Garden Park - filters #4, #7 From a park bench facing the Hudson 0.2 miles WNW of Ground 0 center	$PM_{2.5}$	54.285 mg	47.67 mg 87.8%
A, C	WTC E	9/13/2001	Murray & Greenwich - filters #5, #6 From a window ledge 0.25 miles NNE of Ground 0 center	$PM_{2.5}$	49.919 mg	45.13 mg 90.4%
A, C	WTC F	9/13/2001	Inside 120 Broadway - filters #2, #12 From a marble staircase with no footprints 0.25 miles SE of Ground 0 center	$PM_{2.5}$	53.600 mg	38.73 mg 72.3%
B	WTC 3	9/12/2001	23 Park Row - Ground sample in front of J&R Electronics (across City Hall Park) 0.3 miles E of Ground 0 center	$PM_{<53}$	21.521 g sieved material	sieved - not further fractionated

Control PM Samples

Experiment	Sample Code	Collection Date	Description	Size Fraction	Weight Available	Extracted Wt., % Extracted
C	NIST	1976-1977	NIST Standard Reference Material 1649a (Urban Dust collected in Washington DC)	$PM_{2.5}$	47.984 mg	39.33 mg 82.0%
A	MSH	1980	Mt. St. Helens ash, Washington State, from Graham et al., 1985	$PM_{2.5}$	> 10 g	previously size-separated
A	ROFA	1994	Residual oil fly ash, ROFA Sample 3 from Kodavanti et al., 1998	MMAD: 2.665	> 2 g	milled - not extracted

was gently removed with forceps and excess liquid was drained from the filter into the cup. Filters were placed back in their petri dishes, allowed to dry, and were dessicated before reweighing to determine quantity extracted (i.e. removed) from the filters (Table 1). The suspension of PM was thoroughly mixed, the pH was determined, and 10 ml was pipetted from each of the 7 samples into a single sterilized 150 ml Erlenmeyer flask on ice to make a pooled sample (WTCX). The pH of the pooled sample was also determined. Of the remaining amount from each individual sample, 1 ml was taken for endotoxin analysis, and the remainder was pipetted into sterile 15 ml polystyrene tubes.

The flask containing the pooled sample was covered with parafilm, and the pooled and individual site samples were frozen at -80 °C prior to lyophilization. Holes were poked in the parafilm of the pooled sample, while the caps on the 15 ml individual site sample tubes were loosened. Samples were lyophilized for 2 days at -55 °C and 140 mtorr (Virtis Company, Gardiner, NY). After lyophilization, samples were stored at 4 °C until resuspension in sterile saline on the day of use in oropharyngeal aspiration.

C. Control PM Samples Used in WTC2001 Study

In order to assess toxicity of WTC $PM_{2.5}$, pooled and individual site samples were compared with three other well-characterized $PM_{2.5}$ samples. Standard Reference Materials (SRM) are extensively characterized samples available from the National Institute of Standards and

Technology (NIST, Gaithersburg, MD). SRM 1649a is an urban particulate matter sample which was collected in the Washington DC area in 1976-1977 over a 12 month period and represents a time-integrated sample (NIST, 2001). This material was selected in order to compare toxicity of WTC $PM_{2.5}$ with other typical urban air $PM_{2.5}$ (albeit from an earlier era when leaded gasoline was still in use). Since this material was collected as a total suspended particulate (TSP) sample with a large amount of coarse non-respirable PM, it was necessary to size-fractionate it in order to compare it with the WTC $PM_{2.5}$ samples. Vials of NIST 1649a were purchased and then sent to NYU for size-fractionation using the same procedures as outlined above. The $PM_{2.5}$ fraction was sent back to EPA, and NIST 1649a was extracted from Teflon filters as described above.

The toxicity of WTC $PM_{2.5}$ was also compared to that of a $PM_{2.5}$ fraction of ash from Mt. St. Helens (MSH) in Washington state (Graham et al., 1985). Approximately half of MSH is crystalline in nature, primarily plagioclase, a series of compounds beginning with $NaAlSi_3O_8$ and ending with $CaAl_2Si_2O_8$ which show continuous solid solution from albite to anorthite, with CaAl replacing NaSi as the series progresses. The remaining portion of MSH is amorphous (glass), while there are minor amounts of cristobalite (3%) and quartz (< 1%). The $PM_{2.5}$ fraction of MSH has low toxicity in rats (Raub et al., 1985) and mice (Hatch et al., 1984). Since the MSH sample had already been size-fractionated (Graham et al., 1985), it was not necessary for NYU to further size-fractionate it with their system.

Residual oil fly ash (ROFA) is a fugitive fine PM sample with a high content of bioavailable transition metals including vanadium, nickel, and iron. Numerous studies by investigators at EPA and other institutions have demonstrated that these metals are associated with lung injury in both healthy animals and animal models of cardiopulmonary injury (Dreher et al., 1997; Gavett et al., 1999, Kodavanti et al., 1998, Watkinson et al., 1998). For the WTC2001 study, we chose a sample of ROFA from a boiler system which is toxic yet not as soluble in water as previous samples of ROFA (ROFA sample #3 from Kodavanti et al., 1998), and is therefore more comparable to WTC PM samples which are not extremely water-soluble. The ROFA samples in the study by Kodavanti (1998) were reduced in size by placing each sample with a stainless-steel ball in a stainless-steel cup and shaking vigorously in a ball mill shaker for 30 - 60 minutes, and then passing the sample through a 100 μ mesh nylon screen. ROFA sample #3 has a mass median aerodynamic diameter (MMAD) of 2.665 μ. Although it was slightly larger in size compared with the other samples used in the WTC2001 study, it was decided that further size fractionation at NYU was not necessary. Control PM samples were stored at room temperature in polystyrene or polypropylene tubes shielded from light. See Table 1 for the summary descriptions of control PM samples.

Samples of WTC PM, NIST, MSH, and ROFA were characterized by scanning electron microscopy / energy dispersive X-ray (SEM/EDX), X-ray diffraction (XRF), X-ray fluorescence (XRD), carbon fraction analysis, pH and endotoxin analysis, inductively coupled plasma-mass spectrometry / atomic emission spectrometry (ICP-MS / ICP-AES), and ion chromatography (IC).

D. Physical and chemical analysis of solid (bulk and filter) samples.

1. Scanning electron microscopy / energy-dispersive x-ray (SEM/EDX) analysis. SEM/EDX was used to obtain physical and chemical characteristics of particles and fibers found in bulk WTC2001 and control dust $PM_{2.5}$ samples, and on polycarbonate filters taken during an inhalation exposure using the WTC3 sample. The Personal SEM® (PSEM) (formerly R. J. Lee Instruments, Ltd., now Aspex Instruments, Trafford, PA) was used to conduct the manual, single-particle analyses. The PSEM is a digital SEM/EDX system equipped with secondary and backscattered electron detectors for imaging, and a thin-window EDX detector enabling X-ray detection of carbon and heavier elements. For bulk samples, a small amount was applied to an adhesive carbon tab affixed to an aluminum SEM stub. For filter samples, small pieces (less than 1 cm^2) were affixed to aluminum SEM stubs using a carbonaceous suspension. Images were created using the backscattered electron mode, which enhances the contrast of metals and other heavy elements with the background carbonaceous medium compared with lighter element particles. Photomicrographs of the individual features provide particle morphology and approximate physical size; an x-ray spectrum displayed below the image provides information on the elemental composition of the feature. SEM/EDX analysis was performed by the National Exposure Research Laboratory, Research Triangle Park, NC. Since only 15-30 images were examined from each sample, the results should not be interpreted as quantitative or comprehensive (Mamane et al., 2001). Rather, these qualitative results were primarily used to determine consistency with other analytical techniques described below.

2. X-ray diffraction (XRD) analysis. XRD was used to determine qualitatively whether any crystalline compounds were present in sufficient quantity to be identified in the WTC3 sample used in the inhalation

exposure study. The bulk solid PM_{53} sample was side-drifted into an aluminum holder and mounted into the Siemens D-500 Diffractometer (Bruker Analytical X-Ray Systems, Madison, WI). The generator, set at 45 kilovolts (kV) and 40 milliamperes (mA), generated x-rays from a copper-target x-ray tube. The filament current was 3.46 mA. Intensities were collected by a lithium-drifted silicon detector fitted with a monochromator riding on a goniometer in the coupled $\theta/2\theta$ mode. Peaks were collected in the range $2\theta = 5$ to 85 degrees. Collection software used was Materials Data, Inc. (MDI, Livermore, CA) Datascan, Version 3.2. Evaluation software was MDI Jade 5 using the pattern library Powder Diffraction File (PDF), release 2000 (International Centre for Diffraction Data). XRD analysis was performed by the National Risk Management Research Laboratory, Research Triangle Park, NC.

3. X-ray fluorescence (XRF) analysis. Five polycarbonate filters (Isopore 0.8um #ATTP04700, Millipore Corporation, Bedford, MA) loaded with the WTC3 $PM_{2.5}$ sample used in the inhalation exposure study, along with five lot-matched blank filters, were loaded into liquid-type polyethylene sample cups, placed in a stainless steel sample holder, and analyzed. No film was used to cover blanks or samples in the analysis. X-ray intensities were collected with the Philips PW2404 XRF (Philips Analytical, Inc., Natick, MA), and the loaded particulate was analyzed using the "standardless" software, UniQuant 4, after subtraction of the counts due to the blank filter system (includes filter, polyethylene liquid sample cup and stainless steel sample holder). The intensities were averaged in each channel needed for background subtraction. The blank filter analysis showed slightly elevated counts due to Fe, Cr, Cu, Ca, Cl, S, and Si. The constituents of the dust were evaluated as oxides, but are reported quantitatively as elements with the oxygen stripped. XRF analysis was performed by the National Risk Management Research Laboratory, Research Triangle Park, NC.

4. Carbon fraction analysis. Carbon fraction analysis was used to speciate the carbon content of samples into organic, elemental, and carbonate carbon. Analysis was performed on bulk WTC2001 and control dust $PM_{2.5}$ samples, and on quartz filters taken during an inhalation exposure using WTC3 $PM_{2.5}$. The thermo-optical method, based upon sequential pyrolytic vaporization and detection of the three carbon fractions (Birch and Cary, 1996; Sunset, 2002), was performed by Sunset Laboratory, Forest Grove, OR (bulk samples), and Hillsborough, NC (filter samples).

E. Chemical analysis of liquid extracts of bulk and filter samples.

1. pH. The pH of samples isolated by aqueous extraction was determined immediately after the extraction procedure with an audited calibrated Corning 440 pH meter (audited by Research Triangle Institute, Research Triangle Park, NC).

2. Endotoxin. Aliquots of samples isolated by aqueous extraction were frozen on dry ice and sent by overnight delivery to Associates of Cape Cod, Inc. (Falmouth, MA) for analysis of endotoxin content using the Limulus Amebocyte Lysate (LAL) gel-clot method. LAL-reagent water (lot # 308-331) was used to reconstitute or dilute Pyrotell lysate, endotoxin, and samples, and served as the negative control. Samples were titered using a twofold dilution scheme against control standard endotoxin (CSE; lot #85, Escherichia coli O113, 5 EU/ng). Preliminary inhibition tests (positive product controls) were performed on the undiluted samples spiked with CSE equivalent to twice the sensitivity (λ; 0.03 EU/ml). The error of the gel-clot method is ± one twofold dilution.

3. Inductively coupled plasma - atomic emission spectrometry (ICP-AES) and - mass spectrometry (ICP-MS). WTC2001 $PM_{2.5}$ samples, control dust $PM_{2.5}$ samples, and polycarbonate filters taken during an inhalation exposure using WTC3 $PM_{2.5}$ were extracted with deionized (d.i.) water or 1M HCl, and analyzed for their elemental content. The two extraction liquids are used to estimate easily bioavailable and total bioavailable metal content, respectively. While this speciation scheme is a rough approximation of bioavailability, it has proved useful in characterizing inhalation toxicology endpoints for various source and ambient particulates (Costa and Dreher, 1997; Kodavanti et al., 1998). Milligram-sized aliquots of bulk samples were extracted with 1.6 ml of either liquid. Polycarbonate filters were extracted with 13 ml of either liquid. High-speed centrifugation was used to separate the liquid and solids (17000 x *g* for 1.6 ml samples, 51000 x *g* for 13 ml samples). After dilution, extraction solutions were analyzed quantitatively using ICP-AES (Model P40, PerkinElmer Instruments, Shelton, CT) operated closely following EPA Method 200.7 (EPA, 2002a), and ICP-MS (ELAN 6000, PerkinElmer Instruments, Shelton, CT) operated closely following EPA Method 6020 (EPA, 2002b). Blank Gelman Teflo and Millipore Isopore filters (used in the inhalation study) were run through the extraction procedure. Filter blanks levels for all elements were negligible compared to the levels in the PM samples. Gelman Zefluor and Teflo filters and Millipore Isopore filters are of known, similar

low background levels. These filters are all produced for air particulate sampling and are commonly used for chemical analysis since their background chemical levels are negligible relative to the mass of samples amounts in this study. ICP-AES and ICP-MS analyses were performed by the National Health and Environmental Effects Laboratory, Research Triangle Park, NC.

4. Ion chromatography (IC) of deionized water extracts. Deionized water extracts from the ICP sample prep as described above were analyzed quantitatively for anion and cation content using IC (DX-500, Dionex, Sunnyvale, CA). The AS14 column was used for anion analysis and the CS12 column was used for cation analysis. IC analysis was performed by ManTech Environmental, an onsite contractor for the National Exposure Research Laboratory, Research Triangle Park, NC.

F. Experimental Animals and Weight Randomization.

Young adult (7 week old) female CD-1 mice (an outbred strain) were obtained from Charles River Breeding Laboratory (Crl:CD-1® (ICR) BR) in Raleigh, NC or Portage, MI (the latter used in Experiment A5 only). An outbred strain was chosen because results from any specific inbred strain might be applicable only to that strain. CD-1 mice were selected since researchers in the Experimental Toxicology Division of the U.S. EPA have extensive experience with this strain, while females were chosen for convenience so that they could be housed together in groups corresponding to treatment. The health screening report of mice from the colony accompanied each shipment of animals and was evaluated to determine if there were pathogens detected in the colony which could potentially affect responses. In all shipments, no pathogens were detected which could affect respiratory responses. Mice were housed in plastic cages on beta-chip bedding in groups of 4 per cage in room JJ-4 of the animal colony of the Environmental Research Center, Research Triangle Park, NC. Food (Prolab RMH 3000) and water were provide *ad libitum* and cages were changed at least twice a week. Mice were maintained on a 12 hr light/dark cycle at approximately 22 °C and 50% relative humidity in our AAALAC-approved facility, and held for a minimum of 5 days before treatment. Monthly sentinel screens were negative for sendai, mouse hepatitis virus, mycoplasma pulmonis, CARbacillus, parvovirus, endo- and ecto-parasites, and pinworms. Protocols used in this study were reviewed and approved by the EPA Institutional Animal Care and Use Committee (Laboratory Animal Project Review number 02-03-003 with amendments), and were conducted using national guidelines for the care and protection of animals.

In all experiments, mice were randomly assigned to exposure groups based on weights. The weight randomization program (RandomVB) was developed in-house, validated, and documented in operating procedure OP-NHEERL-H/ETD/IEG/97/18/01 (Animal randomization using a personal computer). The program takes all animal weights and ranks them from lowest to highest. A group mean and standard deviation is calculated for all animals. The number of animals per group and the number of groups is entered. The numbers of animals available at 1, 2, or 3 standard deviations (SD) are calculated. The user then selects the lowest SD which contains the required number of animals for the study. All outliers are eliminated. Additional animals are then eliminated to fit into the required number for the study. Animals are then randomized by weight into the required groups. All animals are accounted for and reasons why they were not selected are displayed. The weight randomization program was used to identify the groups of 4 mice which are housed together in a single plastic cage. Within each cage, mice were individually identified by 1 to 4 marks applied to the base of the tail with a Sharpie permanent ink marker (Sanford, Bellwood IL). Different experimental groups were identified with different colors (e.g. saline control mice - green marks, etc.). In addition the cage cards were marked to identify the experimental group. Marks remained evident for at least two days which was long enough to identify mice at 24 hr termination points. In cases where mice were killed more than 2 days after the initial marking, the tails were remarked where necessary because of excessive fading. Mice were weighed at the time of randomization, again immediately before exposure if randomization occurred before the day of exposure, and whenever one group of mice was killed.

G. Toxicological Endpoints: Experimental Design.

The toxicity of WTC $PM_{2.5}$ samples was assessed in three separate experiments, designated Experiments A, B, and C. Experiment A was designed to study the dose-response characteristics of the pooled sample of WTC PM (WTCX) in comparison with ROFA (toxic control), MSH (low toxicity control), and saline vehicle control. Experiment B was designed to study the responses associated with nose-only inhalation exposure of WTC3 $PM_{2.5}$ in comparison with the responses of mice exposed to air only. Experiment C was designed to compare toxic responses of WTC $PM_{2.5}$ from individual sites with each other and with NIST 1649a.

In all experiments, a group size of 8 was selected

based on scientific judgement and experience with the typical variability of collected data (except the first part of experiment A, where n = 12; see Results - Experiment A - BAL parameters for explanation). Endpoints were analyzed in a total of 388 mice in all 3 experiments. In general, the endpoints were chosen to assess pulmonary function impairment, lung injury and inflammation, and pathological manifestations of respiratory tract injury. Experiments utilizing oropharyngeal aspiration (A and C) were emphasized over inhalation experiments (B) for several reasons: 1) the quantities of samples available for study were generally limited, and aspiration requires much less material (10-100 mg) than inhalation (10 g preferred); 2) aspiration delivers a precise quantity of PM to the lung at a specific time point, while the inhaled dose is more difficult to predict or quantify; 3) inhalation exposure studies are labor intensive and therefore fewer comparative analyses of the WTC $PM_{2.5}$ could be accomplished in the available time frame compared with studies utilizing oropharyngeal aspiration, and 4) oropharyngeal aspiration (equivalent to intratracheal instillation) is specifically recommended in evaluation of panels of test materials for their relative potential to produce toxicity (Driscoll et al., 2000).

1. Experiment A. In 5 sub-experiments, groups of female CD-1 mice were exposed to pooled WTC $PM_{2.5}$ sample X (10, 31.6, or 100 μg), MSH (100 μg), ROFA (10 or 100 μg), or saline vehicle control by oropharyngeal aspiration on day zero. The dose of 31.6 μg represents the half-log difference between 10 and 100 μg (i.e. $10^{1.5}$ = 31.6). The high dose of 100 μg was selected based on our experience that at this dose nearly all PM samples will induce at least a mild inflammatory or physiological response; any sample that does not induce any response at all at this dose can be judged to possess low toxicity. Doses higher than 100 μg in the mouse may be of questionable relevance due to the potential for artifactual local inflammatory responses in response to bolus administration (Driscoll et al., 2000). Four mice per sample group were tested within each sub-experiment (n = 28 per sub-experiment; total experiment A: n = 140).

In sub-experiments A1, A2, and A5 (total n = 12 mice per sample group), airway responses to aspiration of the PM samples was assessed by comparison of breathing parameters just before and after aspiration (see method below). On day 1, diffusing capacity of the lung for carbon monoxide (DLCO) was assessed, and mice were then killed and bronchoalveolar lavage (BAL) fluid cells, proteins, and enzymes were recovered and quantified to assess lung injury and inflammation.

In sub-experiments A3 and A4 (n = 8 mice per sample group), airway responsiveness to methacholine (Mch) aerosol was determined on day 1. Mice were then killed, and lungs were removed and fixed for histopathological assessment. Airway hyperresponsiveness to nonspecific bronchoconstrictive agents such as Mch is a primary feature of asthma (Sears, 1997) as well as reactive airways dysfunction syndrome (RADS) which develops after high-level occupational exposure to irritant gases, fumes, or smoke (Gautrin et al., 1999). Induction of this condition by PM in nonallergic normal mice can be considered as a marker of respiratory tract injury.

2. Experiment B. Two groups of female CD-1 mice were exposed in nose-only inhalation exposure tubes one time to a $PM_{2.5}$ sample (WTC3) or air only for 5 hr (n = 48 per exposure group; total experiment B: n = 96). This WTC3 sample was derived from a sieved but not previously fractionated PM_{53} sample of WTC3 by aerodynamic size-separation during exposure. Although some irritant responses are transitory and therefore would be best measured during exposure (Costa and Schelegle, 1999), we do not currently possess the recently developed technology (e.g. Buxco Electronics, Sharon, CT or CH Technologies, Westwood, NJ) which allows some respiratory parameters to be measured during nose-only exposures. Therefore, breathing parameters were compared just before and after inhalation exposure (n = 12 per group). On days 1, 3, and 6 after the exposure, 16 mice from each group were assessed for DLCO and BAL parameters (n = 8) or responsiveness to Mch aerosol and lung and nasal histopathology (n = 8).

3. Experiment C. In 2 sub-experiments, groups of female CD-1 mice were exposed by oropharyngeal aspiration to 100 μg of PM from one of 7 individual WTC sample sites, to 100 μg of NIST 1649a (referred to as NIST hereafter), or to saline vehicle only. In sub-experiment C1, mice were exposed to WTC8, WTC13, WTCF, NIST, or saline. In sub-experiment C2, mice were exposed to WTC11, WTCB, WTCC, WTCE, or saline. On days 1 and 3, mice were assessed for responsiveness to Mch aerosol, BAL parameters, and lung histopathology (n = 8 per group per time point, except saline sub-experiment C2: n = 4 per time point; total sub-experiment C1: n = 80, total sub-experiment C2: n = 72, total experiment C: n = 152).

H. Oropharyngeal Aspiration of PM Samples.

A Sartorius model AC211S analytical balance (Edgewood, NY; audited by Research Triangle Institute, Research Triangle Park, NC) was used to weigh PM samples for oropharyngeal aspiration. The operation of the balance was tested by weighing calibrated weights

before and after weighing samples each day (Class U calibrated weights, Denver Instrument Company, Arvada, CO). PM samples were allowed to come to room temperature from 4 °C before weighing. Sterile 2.5 ml glass vials or 5 ml polystyrene snap cap vials were used to weigh and resuspend PM samples. Vials weights were tared, and a sterilized stainless steel spatula was used to transfer sample to the weighing vial, and the sample was weighed. The sample was then resuspended with sterile saline (Sigma S-8776 single use vials, lot 128H2310) using a calibrated Rainin Pipetman at a concentration of 2 mg/ml. All mice aspirated a volume of 50 µl. Samples were vortexed and used straight (100 µg dose) or diluted as necessary (to 0.632 mg/ml or 0.2 mg/ml for 31.6 µg or 10 µg doses, respectively). All samples were sonicated for 2-4 minutes at 22 °C (Branson model 3210R-DTH, Danbury, CT) prior to oropharyngeal aspiration.

Mice randomized into different exposure groups were anesthetized in a 2.7 L plexiglass chamber by passing house air through an aerator containing methoxyflurane (Metofane; Mallinckrodt, Mundelein, IL). The vapor induced rapid anesthesia, at which time the mouse was taken out of the chamber and placed on an aspiration platform. The tongue was gently pulled back and held with a forceps, and 50 µl of PM suspension or saline alone was pipetted in the back of oropharyngeal region using a 200 µl tip. The tongue was held until the animal was forced to aspirate the sample, and placed back in its cage. Mice recovered within 5 or 10 minutes of this procedure. This technique is equivalent to intratracheal instillation in deposition efficiency (Foster et al., 2001), and several publications describe experiments in which it was successfully used (e.g. Dreher et al., 1997, Gavett et al., 1999, Kodavanti et al., 1998).

I. Nose-Only Inhalation Exposure.

In order to assess the effects of WTC $PM_{2.5}$ on upper respiratory tract responses, mice were exposed to WTC3 or air only in two separate nose-only inhalation exposure chambers. The exposures were conducted for 5 hours in 52-port nose-only flow-by inhalation chambers (Lab Products) on November 27, 2001. The exposure time was based on practical considerations of the tasks involved on the exposure day. The WTC3 sample was a tan powder received in a plastic jar, and was desiccated at room temperature prior to use. Preliminary exposures were conducted on several days prior to exposure to set exposure parameters, which indicated that an aerosol concentration of 10-15 mg/m^3 could be achieved. The control chamber and the WTC3 chamber had similar flow rates (~ 12 L/min) and received air from the same source. The aerosol was generated using a unique exposure system which conserves sample by using a carpenter's chalk line to pick up particles from a small Tygon tube dust reservoir (illustrated in Ledbetter et al., 1998). The dust is carried out through an orifice and blown off the string in a discharge head with a high velocity air jet. The particles are carried through a particle charge neutralizer and 2.5 µ cut-point cyclone to remove particles larger than $PM_{2.5}$, and finally enter the inlet of the nose-only chamber.

Nose-only exposure tubes were constructed from 50 ml polypropylene centrifuge tubes with the bottom end removed. Mice were randomized into exposure groups as described above, and 49 in each group were placed in exposure tubes (1 extra per group in case any mice died during the exposure). Mice were not acclimated to the tubes prior to exposure, since stress may be an important component of the response to WTC PM. In order to measure immediate airway responses to air or WTC3 sample and also to handle the large number of mice, the control air exposure was begun 1 hour before the WTC3 exposure.

Dust concentration was determined gravimetrically on 5 Teflon filters (45 mm diameter with 1 µ pore size) taken at a sample flow rate of approximately 0.24 L/min. The filters were weighed just prior to and after sampling using a Cahn C-30 balance housed in a controlled temperature and humidity enclosure. Real-time PM concentration was achieved with an aerosol monitor (Dust Track, TSI Inc., St. Paul, MN) on the chamber exhaust. The particle size was determined gravimetrically using a Mercer Cascade Impactor (Intox Products, Albuquerque, NM). On November 29, 2001, the exposure system was restarted and 7 Teflon and 3 polycarbonate filters were taken for chemical analysis. On December 17, 2001, 18 polycarbonate filters (9 WTC3 and 9 blanks) were collected for chemical analysis and 3 quartz filters were collected for carbon analysis.

J. Respiratory Responses Assessed by Whole Body Plethysmography.

1. Immediate Airway Responses to $PM_{2.5}$ Exposure.

Exposure to $PM_{2.5}$ by oropharyngeal aspiration or by inhalation may result in immediate changes in breathing parameters. Breathing parameters in unanesthetized unrestrained mice were assessed in a 12-chamber whole body plethysmograph system (Buxco Electronics, Sharon, CT). The animal chambers have a pneumotach on the roof to measure pressure (which is proportional to air flow) relative to the pressure in a reference chamber vented to the atmosphere. The breath by breath signals are taken by the program software to compute respiratory rate

(frequency, *f*, breaths / min) and other parameters including enhanced pause (PenH). Although PenH is at best an indirect measure of flow resistance, it does correlate well with lung resistance and reflects changes occurring during bronchoconstriction (Hamelmann et al., 1997), although other responses such as mucus production may increase PenH. The convenience of rapidly measuring respiratory parameters in twelve mice at once was a major consideration in utilizing this technique rather than the double plethysmograph or tracheotomized ventilated methods which allow direct measures of airways resistance and compliance, but are time and labor intensive. A protocol was written to record and average baseline measurements of mice in calibrated chambers for 10 min, pause for oropharyngeal aspiration (or stop during inhalation exposure), and then resume recording measurements for one hour. The time between oropharyngeal aspiration and monitoring of responses was approximately 6 minutes, while about 20 minutes was needed after inhalation exposure to remove mice from exposure tubes, weigh them, and transport them to the plethysmograph chambers. PenH was automatically calculated by the software (and confirmed by examination of random data) using expiration time (Te), relaxation time (RT), and peak expiratory and inspiratory flows (PEF, PIF) according to the following expression: PenH = [(Te-RT)/RT] x [PEF/PIF]. Examination of the data after exposure showed that utilization of the first 10 or 15 minutes of the data was not more sensitive in detecting changes in respiratory parameters than the entire hour of post-exposure monitoring, and therefore responses over the whole post-exposure hour were utilized and averaged. The percent change in *f* and PenH after exposure to PM was expressed as [(Post-value - Pre-value) / Pre-value] x 100%.

2. Airway Responsiveness to Methacholine Aerosol.

Airway responsiveness to increasing concentrations of aerosolized methacholine (Mch) was measured in mice in calibrated chambers. After measurement of baseline PenH for 5 minutes, saline or Mch in increasing concentrations (4, 8, 16, 32, and 64 mg/ml) was nebulized through an inlet of the chamber for 1 min. The aerosol drier was automatically turned on immediately after the aerosolization period for 2 min. Measurements of PenH and other parameters were continued for an additional 1, 2, 3, 4, 8, and 12 minutes after saline or increasing doses of Mch, for a total time of 4, 5, 6, 7, 11, and 15 minutes (0, 4, 8, 16, 32, 64 mg/ml Mch, respectively). One minute pause periods between aerosolizations allowed time to change solutions for nebulization. After subtracting baseline values from responses to saline or Mch, the area under the curve (PenH AUC; PenH - sec) for these recording intervals was calculated using the trapezoid method.

K. Diffusing Capacity of the Lung for Carbon Monoxide.

The ability of the lungs to allow diffusion of gases (O_2, CO_2) across the alveolar-capillary barrier is dependent on physical properties of the gases and the alveolar-capillary membrane, and may be limited by perfusion or diffusion (Levitzky, 1995). Diffusion limitation may be caused by thickening of the alveolar-capillary barrier (e.g. by interstitial or alveolar edema). Diffusion of carbon monoxide (CO) is limited only by its diffusivity in the barrier and by the surface area and thickness of the barrier. The diffusing capacity of the lung for CO (DLCO) is therefore a useful test of the integrity of the alveolar-capillary membrane (Levitzky, 1995).

To determine DLCO rapidly and increase sensitivity from individual mice, 4 mice were placed together in a single 7.8 L bell jar associated with a Pharmacokinetic Uptake System (consisting of an oxygen monitor, flow meter, pump, pressure gauge and transducer, mass flow controller, and computerized data collection and control system). Approximately 6.6 ml of research grade CO (99.99%) was injected into the system. The initial concentration of CO in the chamber was approximately 700 ± 10 ppm. CO concentrations were taken every 15 seconds (Bendix Model 8501-5CA CO Analyzer), and continued for approximately 10 minutes. Temperature, humidity, airflow, pressure, and oxygen were monitored during the test. The DLCO is expressed as the slope of the fitted line of [CO] vs. time (ppm/min).

L. Bronchoalveolar Lavage (BAL).

Mice were anesthetized with urethane (1.5 g/kg i.p.) and killed by exsanguination via severing the renal artery. The trachea and lungs were exposed and a 20 g catheter was sutured into the trachea. Mice were lavaged with two aliquots of Ca^{2+}, Mg^{2+}, and phenol red-free Hanks' balanced salt solution (HBSS; 35 ml/kg, Life Technologies, Bethesda, MD). Approximately 85% of the total instilled volume was recovered in all treatment groups. The BAL fluid was maintained on ice and centrifuged at 360 x g for 10 minutes at 4 °C. Supernatants were transferred to a separate tube in order to prepare aliquots for biochemical analyses. BAL cells were resuspended in 1 ml of HBSS and counted (Coulter Z1, Hialeah, FL). Cytospin preparations of BAL cells were made for each sample and stained with Wright's Giemsa using an automated slide stainer (Hematek 2000,

Elkhart, IN). Cell differentials were performed by one person (SHG) counting 500 cells per slide. After lavage, the lungs were removed and stored at –80° C for future assays (to be determined).

Assays for total protein, albumin, lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAG) are routine measures of lung injury (Henderson et al., 1985) and were carried out on an aliquot of BAL supernatant as previously described using a Cobas Fara II centrifugal spectrophotometer (Gavett et al., 1997). Four other BAL supernatant aliquots were prepared from each sample; one of these was supplemented with 10% fetal bovine serum to prevent loss of cytokines and other proteins in low protein concentration fluids, and the 4 aliquots from each sample were stored at –80° C. These samples are available for analysis of cytokines and other proteins (to be determined).

M. Histopathology

*1. **Lung histopathology.*** In experiments A and B, mice which were tested for Mch responsiveness were subsequently assessed for lung histopathology, while in experiment C, all mice were tested for Mch responsiveness and were lavaged before assessment of lung histopathology. Mice were anesthetized with urethane and killed as described above for BAL. Lungs were removed and fixed by tracheal perfusion in a fume hood with ice cold 4% paraformaldehyde at 25 cm pressure for 15 minutes. The trachea was then tied off and placed in a vial of 4% paraformaldehyde at 4 °C. After 24 hours, the lungs were drained and placed in phosphate buffered saline at 4 °C.

The lungs were transferred to Experimental Pathology Laboratories (Research Triangle Park, NC), where fixed lungs were processed to paraffin blocks, sectioned at an approximate thickness of 5 μ, placed on glass slides and stained with hematoxylin and eosin (H&E). Longitudinal coronal sections were cut on a lateral plane to include mainstem bronchi for viewing a maximal amount of lung area. Two additional unstained lung sections were prepared for future use. Histopathologic observations for individual animals in each experiment were tabulated, and the degree of severity of inflammatory changes and the presence of PM-related pigment were graded on a scale of one to five (1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high). The pathologist knew which animals comprised a group, which group was the saline or air-exposed control group, the day after treatment, and the doses given to the experimental groups, but did not know the identities of the individual PM samples other than by a unique number or letter.

*2. **Nasal histopathology.*** Dr. James Wagner of Michigan State University (MSU) instructed EPA personnel in this procedure, utilized on mice from the nose-only inhalation exposure (experiment B). Immediately after death, the head of each animal was removed from the carcass and both nasal passages were fixed by slowly flushing retrograde through the nasopharynx with 1-2 ml 4% paraformaldehyde. The nasal cavity was then immersed in a large volume of the fixative for at least 24h until further processing. The fixed nasal cavities were placed in 0.1 M PBS (pH 7.2, 4 °C) and shipped overnight to Dr. Wagner at MSU. Nasal cavities were decalcified in a 13% solution of formic acid for 5 days, and then rinsed in distilled water for 1h. After decalcification, three transverse tissue blocks of the nasal cavity, cut perpendicular to the hard palate, were selected for light microscopic analysis. The first tissue block was sectioned from the proximal aspect of the nasal cavity immediately posterior to the upper incisor tooth (T1). The second transverse tissue block was taken at the level of the incisive papilla (T2) and the third and most distal tissue block was taken at the level of the second palatial ridge (T3). The tissue blocks were embedded in paraffin, and 6 μm-thick sections were cut from the anterior surface of each block. Sections were histochemically stained with hematoxylin and eosin for morphologic identification of nasal tissues. Nasal tissues (three sections/mouse) from a total of 48 mice tested for Mch responsiveness (8 mice/exposure group/time point) were microscopically examined by Dr. Jack Harkema (MSU). Nasal lesions were graded on the following scale: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked inflammation.

N. Statistical Analysis

All statistical analysis were done using SAS procedures (Cary, NC). There were generally three types of responses collected: 1) PenH responses recorded repeatedly for each animal as area under the curve (AUC) for various concentration exposures to Mch; 2) responses to DLCO were analyzed from a single response from 4 animals; and 3) individual measurements measured once for each animal as a univariate variable. Experimental designs varied with each experiment and each part of an experiment. Statistical designs used were replicated completely random designs for experiment A. Crossed-designs were used for experiment B and C involving treatments (TRT) and days (DAY). Randomized block designs were used for DLCO experiments.

When initial multivariate repeated measures analysis of variance (MANOVA) test showed significant interactions between dose of Mch and TRT or DAY in the

airway responsiveness studies, univariate linear regression was used in all subsequent tests. The models used in these regression studies were analysis of covariance (COV) with tests for parallelism for each TRT and DAY combination. In experiment A, natural logarithms were used for both Mch concentration (C) and PenH AUC responses. The linear regression Log (PenH AUC) = a1 + b(Log C) reduces to a power function of the form PenH = $a_2 \cdot C^b$. No logarithms were used in Experiments B and C due to several negative values resulting after baseline adjustments. Techniques similar to ordinary stepwise regression were used in COV analyses. Overall test of parallelism of regression lines was done first. Subgroups of the TRT and DAY combinations were determined to get subgroups exhibiting a common slope. Within a subgroup with a single slope, subsequent tests were done to determine if the means were different (using individual contrast tests). Body weight was determined repeatedly for animals. Due to animals being removed and killed, numbers of mice in each group were different on different days after exposure. For days when few animals weights were determined, univariate analysis of variance (ANOVA) was used to test for TRT effects. For those days where most of the weight data occurred, MANOVA techniques were used for statistical tests.

For each TRT and DAY combinations with a univariate response, a determination was made if the variances could be considered homogeneous. If the variance ratios were greater than 10-fold then all of the responses were ranked from smallest to largest across all TRT and DAY combinations. Then ranks replaced the original responses for the univariate ANOVA. Sometimes the variances of the ranks for TRT and DAY combinations still indicated heterogeneity. Then additional judgment was used to help insure that this heterogeneity of variance did not affect the overall conclusions. In experiment A, a replicate was called DAY. When replication was shown to have no significant contribution in the ANOVA results, DAY was not included in subsequent ANOVAs. For responses with many "zero" values, the residuals from the ANOVA were plotted and analyzed by univariate techniques to determine if the residuals generally met the assumptions required for ANOVA. When interactions between TRT and DAY occurred, these were pointed out and in some cases further ANOVA were done for each DAY. When ranks were used for the response, the ranks were regenerated for each day separately. When TRT was significant, follow-up comparisons of means were done using Tukey's multiple comparison tests.

The statistical tests examined only whether groups were significantly different from each other. In the reporting of the results, for the sake of brevity, groups are sometimes referred to as having significantly greater values than other groups. These statements should be read as groups are significantly different from each other, and the mean of one group is greater than the mean of another.

III. Results

A. Chemical analysis of solid samples and liquid extracts.

1. Endotoxin and pH levels. The pH of water-extracted WTC $PM_{2.5}$ ranged from 8.88 in WTCE to 10.00 in WTC8 (Table 2). The alkaline pH is consistent with previous reports of WTC PM (USGS, 2002) and probably results from the building materials comprising much of the dust (see below). The pH of lyophilized WTC $PM_{2.5}$ reconstituted in unbuffered saline was very close to neutral, while MSH was very slightly acidic and ROFA was moderately acidic (average 3.74 at 2 mg/ml). It is not known why the pH of WTC $PM_{2.5}$ should be close to neutral after reconstitution in saline; perhaps the salt neutralizes a basic component of the extract. Endotoxin levels in WTC $PM_{2.5}$ samples were minimal in comparison with other urban PM samples such as NIST 1649a, which was also low (Table 2). Several thousand times this level of endotoxin caused an acute neutrophilic response in the lungs of CD-1 mice (Dhingra et al., 2001). The level of endotoxin in the samples used in this study would not be anticipated to contribute directly to any inflammatory response in the lungs.

2. Elemental and Ion Analysis. The ICP data showed that water-soluble calcium and sulfate content amounted to 56-63% of the WTC $PM_{2.5}$ samples (Table 3). In general, the elemental and ion compositions were consistent among the different samples tested. ICP data for the 1M HCl-soluble extracts of WTC $PM_{2.5}$ showed an additional 1-2 weight percent calcium content. This increase may be attributed to calcite or other water-insoluble calcium salts which are soluble in 1M HCl (see below for data on compound analysis). There was no evidence of stainless steel contamination from the forceps used to handle the WTC PM filters or from the stainless steel balls used to size-fractionate the ROFA sample.

The ICP results for the aerosolized $PM_{2.5}$ cut fraction of WTC3 generally agree well with those determined by XRF (Table 3). Calcium content of acid-extracted WTC3 was somewhat lower by ICP (20-22%) than calcium content of solid WTC3 by XRF (26.6%). This may reflect an incomplete extraction in the one hour timeframe of sample preparation method for ICP, or the presence of other insoluble forms of calcium in the WTC3. The XRF values are higher for most other elements, which reflects the incomplete dissolution of the WTC3 matrix by the weak (water) and moderate (1M HCl) extraction liquids. Elements such as magnesium and zinc, which exist in compounds more amenable to acid dissolution, agree more closely (Weast, 1985; Budavari, 1996). Elements such as aluminum, iron, and titanium, which are in the form of

Table 2. Endotoxin and pH Levels of PM Samples after Water Extraction and Resuspension in Saline.

Sample Code [a]	pH in water	Endotoxin [d] EU/ml	Endotoxin [d] Inhibition	pH in Saline [e]
Water [b]	5.28			
WTC 8-100	10.00	0.50	none	
WTC 11-100	9.16	0.25	none	
WTC 13-100	9.47	0.50	none	
WTC B-100	9.54	0.25	none	
WTC C-100	9.32	0.50	none	
WTC E-100	8.88	0.25	none	
WTC F-100	9.55	0.50	none	
NIST-100 [c]	4.20	25	none	
Saline				6.67
WTCX-10				7.38
WTCX-31.6				7.38
WTCX-100	9.35			7.36
MSH-100				6.61
ROFA-100				3.74

[a] WTCX indicates pooled sample of WTC8, WTC11, WTC13, WTCB, WTCC, WTCE, and WTCF. "-100" indicates 100 µg/50 µl dose = 2 mg/ml. "-31.6" indicates 31.6 µg/50 µl dose = 0.632 mg/ml. "-10" indicates 10 µg/50 µl dose = 0.2 mg/ml. Water-extracted PM samples were lyophilized and resuspended in sterile saline.

[b] Water used to extract filters.

[c] NIST Standard Reference Material 1649a (Washington DC TSP PM).

[d] Endotoxin levels measured as endotoxin units (EU) per ml water extract. Samples were tested for inhibition of the endotoxin assay (none was detected).

[e] Average of 3-4 measurements.

Table 3. Elemental and Ion Analysis of WTC2001 Samples [a]

Sample:	WTC 3			WTCX (pooled)		WTC B	MSH		ROFA		NIST
Diluent:	DI H_2O	1M HCl	none	DI H_2O	1M HCl	DI H2O	DI H2O	1M HCl	DI H2O	1M HCl	DI H2O
No. Analyses:	4	4	5	2	1	1	2	1	2	1	1
Analyte											
SO_4^{-2}	376488.6	344439.8	375300	439120.3	379429.4	432570.7	955.2	973.4	274962.0	242277.8	86725.3
Ca	184904.4	218019.2	265600	187493.7	196745.9	183794.1	351.5	1970.8	18590.8	19663.0	12649.5
Si			30000								
Al	1346.3	4072.2	9930	555.7 (*)	1476.8	537.1 (*)	48.4 (*)	1260.4	6739.8	8604.3	1049.1
Mg	1112.9	5414.1	6550	651.5	2257.6	354.1	37.3	1250.2	24895.8	24655.2	1140.7
Fe	150.0 (*)	2450.4	6290	6.7 (*)	1098.5	6.0 (*)	-0.1 (*)	1833.4	763.0	19512.4	407.5
Cl^-	**2851.0**		3330	**2699.0**			**1103.0**		**302.0**		**1164.0**
K			2690								
Zn	22.7	1413.1	1760	13.4	410.1	4.1	-0.0 (*)	3.7	6555.9	5932.3	503.9
Ti	12.1	180.9	1450	5.2	41.6	4.0	1.3	17.6	2.6	137.5	1.8
Na^+	**1290.0**		725	**1139.0**			**721.0**		**44179.0**		**2153.0**
NO_3^-	**938.0**			**6496.0**			**0.0**		**0.0**		**7390.0**
PO_4^{-3}	**0.0**		779	**0.0**			**0.0**		**0.0**		**3317.0**
F^-	**406.0**			**799.0**			**0.0**		**352.0**		**466.0**
Pb	2.2	141.3		0.7	33.4	0.4	0.0 (*)	0.5	17.9	789.6	1378.7
Mn	4.5	107.9		1.8	24.0	0.7	3.8	38.4	365.0	458.6	77.2
Cu	5.5	76.0		6.4	21.8	4.7	0.6	6.8	572.8	628.4	83.6
Ba	25.6	75.6		8.7	31.0	7.2	0.1 (*)	5.0	3.0	57.5	11.2
Sb	17.7	43.8		9.4	17.4	7.0	0.0 (*)	-0.0 (*)	1.5	146.5	3.5
NO_2^-	**38.0**			**0.0**			**0.0**		**0.0**		**0.0**
Mo	1.0	33.7		1.8	3.2	4.9	0.1	0.1	0.0 (*)	339.3	1.8
Ni	2.6	19.7		4.1	3.1	1.8	0.0	0.9	17027.7	16988.2	32.2
Sn	0.8	12.1		1.3	3.1	0.1 (*)	0.0 (*)	0.0 (*)	0.0 (*)	51.0	0.1 (*)
Cr	10.0			1.4 (*)		1.2 (*)	0.1 (*)		6.1		6.1
Cd	0.3	7.2		0.7	1.9	0.3	0.0	0.0	8.9	11.9	19.9
Be	-0.0 (*)	2.7 (*)		0.3 (*)	0.2 (*)	-0.1 (*)	0.0 (*)	0.0 (*)	1.4 (*)	1.7 (*)	0.1 (*)
Co	0.2 (*)	1.6		0.8	0.9	0.1 (*)	0.0 (*)	0.4 (*)	495.0	510.4	2.1
As	1.3	-10.4 (*)		1.1	-0.4 (*)	0.3	0.1	0.2	1.7	106.4	13.5
Tl	0.1 (*)	-25.5 (*)		0.5	-1.2 (*)	0.1 (*)	0.0 (*)	-0.1 (*)	0.0 (*)	-0.2 (*)	0.3
V									1748.6	35693.6	
NH_4^+	**0.0**			**0.0**			**0.0**		**0.0**		**25355.0**
Total µg/g:	569632	576475	704404	639019	581598	617299	3223	7362	397593	376566	143953
% Total Mass:	57.0	57.6	70.4	63.9	58.2	61.7	0.3	0.7	39.8	37.7	14.4

[a] Results shown are average values for number of analyses indicated, expressed as µg soluble analyte / g solid sample extracted, for deionized (DI) water and 1M HCl extractions. Analyte concentrations were determined by ICP-MS, except underlined values which were determined by ICP-AES, bold values which were determined by ion chromatography, and column labelled none, where solid sample was analyzed by XRF (indicated by heavy solid-line box). (*) Value below detection limit. Analytes are arranged in order of decreasing content in WTC 3 sample, by whatever analysis provided highest content.

complex oxides usually in combination with silicon, are much less soluble under the acid extraction conditions used in this study, and do not agree as well (Weast, 1985; Budavari, 1996). The weight-percent ratios of silicon, aluminum, magnesium, and iron are in the proportion of those found in portland cement, a major component of concrete (NIST, 2002; McKetta, 1978).

Comparison of the water-soluble transition and heavy metal content of the WTC PM samples with the control dusts shows the overall metal level trend as Mt. St. Helens < WTC2001 < NIST < ROFA. ROFA has high levels of water-soluble transition metals including vanadium,

Table 4. Carbon fraction analysis of PM Samples in WTC2001 Study [a]

Sample:	WTC3	WTCX (pooled)	WTC B	MSH	ROFA	NIST SRM 1649a
% Carbon Fraction:						
Organic	6.88	0.93	2.11	0.06	1.31	10.82
Elemental	0.31	0.00	0.01	0.07	13.63	15.10
Carbonate	1.39	0.60	0.35	0.00	1.32	0.00
Total	8.58	1.53	2.47	0.13	16.26	25.92

[a] Organic, elemental, and carbonate carbon fractions were analyzed as described in text. Results are expressed as percent of total mass of sample.

Table 5. XRD Analysis of Compounds Present in WTC 3 Sample [a]

ICDD Number	Formula	Mineral Name	Relative Amount
05-0586	$CaCO_3$	Calcite	Major
33-0311	$CaSO_4 \cdot 2H_2O$	Gypsum	Major
41-0224	$CaSO_4 \cdot 0.5H_2O$	Bassanite	Minor
46-1045	SiO_2	Quartz	Minor

[a] Analysis showed about half crystalline materials (50.6% above background), and the remainder was amorphous. After smoothing and subtracting background, evaluation software (MDI Jade 5) was used to match patterns with library available from International Centre for Diffraction Data Powder Diffraction File, release 2000.

nickel, and iron which are important in its toxicity (Kodavanti et al., 1998). The 1M HCl-soluble metal content trend is Mt. St. Helens < WTC2001 < ROFA (not enough NIST sample was available to run the test).

3. Carbon analysis. The WTC2001 samples had low total carbon content, in the range of 1.5-8.5% (Table 4), in comparison with control samples such as NIST (26%) and ROFA (16%). MSH had almost no carbon, as expected from this crustal PM sample. The WTC3 sample used in the inhalation study had about 4 times as much carbon as the other two WTC samples. This result may be due to differences in the method by which the samples were isolated (physical separation vs. aqueous extraction and lyophilization) or may simply be due to variability in carbon content of samples from different locations. Despite the variation in total carbon content of WTC PM samples, the ratios of elemental, organic, and carbonate carbon were similar. Elements not listed in Table 3 or 4 (~30% of total mass) are likely O and H from adsorbed water and O, H, and N from organic or inorganic compounds.

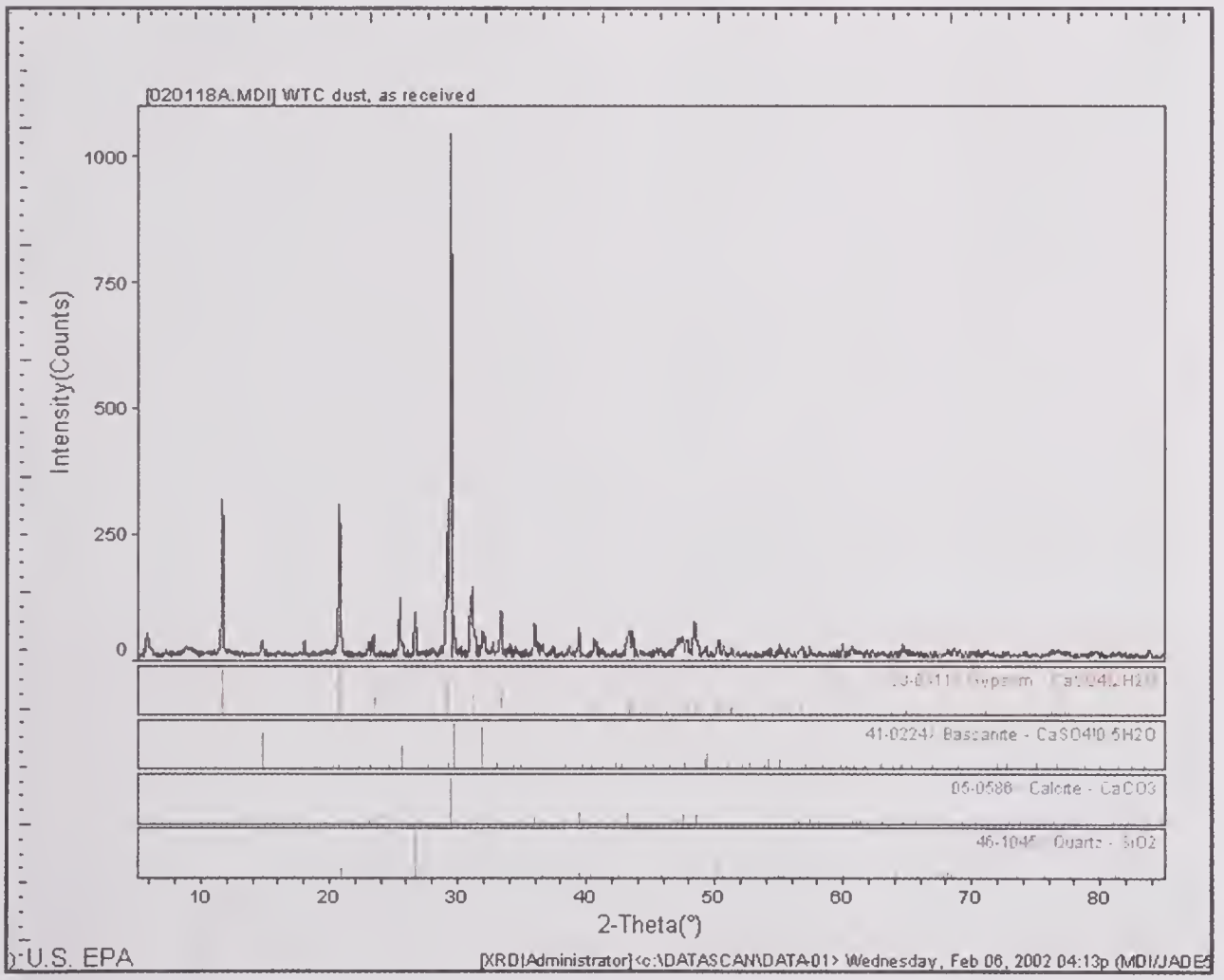


Figure 3. X-ray diffraction (XRD) analysis of WTC 3 sample (PM_{53}) used in nose-only inhalation exposure study (Experiment B). Peaks were collected in the range, $2\theta = 5 - 85°$. Collection software used was Materials Data, Inc. Datascan, version 3.2

4. Compound analysis by XRD. XRD analysis of WTC3 PM_{53} (before size segregation by the inhalation exposure system) showed a complex pattern containing 25 peaks, indicating the presence of several crystalline materials. The peak area above the background curve was 50.6%. The 49.4% below the curve indicated that WTC3 consisted of about half amorphous materials. Four patterns were identified as being consistent with peaks identified in the dust. Figure 3 shows the XRD spectra of WTC3 and those of the matched compounds. Two compounds were identified as major constituents (calcium carbonate (calcite) and calcium sulfate dihydrate (gypsum)), and two were identified as minor constituents (bassanite and quartz, Table 5). The XRD data are

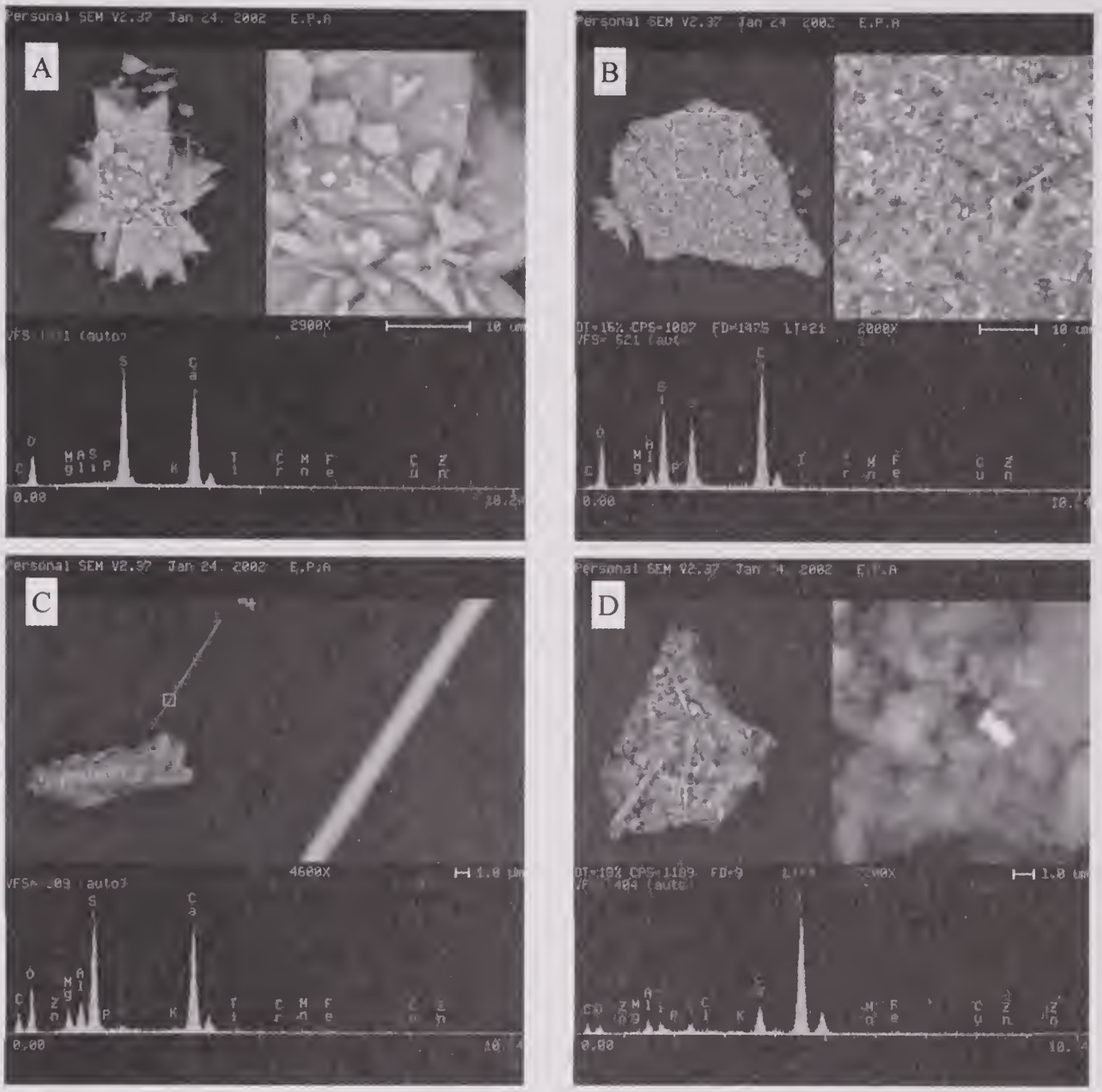


Figure 4. SEM/EDX results from water-extracted lyophilized WTC PM samples. The upper-left quadrant of each photomicrograph shows a field of view with the particle of interest within the smaller square in that field. The upper-right quadrant shows a zoomed-in view of the feature (the area from within the square in the upper-left quadrant), and the lower half shows the elemental spectrum acquired with the electron beam centered on the small (barely visible) square in the zoomed-in view. A. Example of Ca-S crystal which dominated the samples. B. Example of fine particle aggregate which was prominent in the samples. C. Example of fiber found in the samples. D. Example of metallic particle within fine particle aggregate.

consistent with the ICP data which show water-soluble calcium and sulfate in the same proportions as gypsum. Gypsum is completely water-soluble at the solid/liquid ratio of the extraction conditions used in the ICP analysis, while calcite is not water-soluble. The sample of MSH was also analyzed by XRD and the results were consistent with those previously reported (Graham et al., 1985; data not shown).

5. SEM/EDX analysis. Water-extracted and lyophilized WTC PM samples were dominated by snowflake-like crystals composed of calcium and sulfur (Figure 4A). Aggregates of fine particles composed of various combinations of Mg, Al, Si, S, and Ca were also prominent (Figure 4B). Fibers approximately 1 μm in diameter were found in most of the samples and had a composition similar to the fine particle aggregates (Figure 4C). Metallic particles (mostly Ti and Fe, though Zn, Pb, Ba, and Cu were also found) were found typically as inclusions in the large fine particle aggregates (Figure 4D). The crystals and aggregates were likely not original to the bulk sample but were formed as a result of the aqueous extraction process.

SEM/EDX analysis of the aerosolized $PM_{2.5}$ cut fraction of WTC3 showed the same overall chemistry as the extracted and lyophilized WTC PM samples: the majority of particles were composed of Ca or Ca-S, some

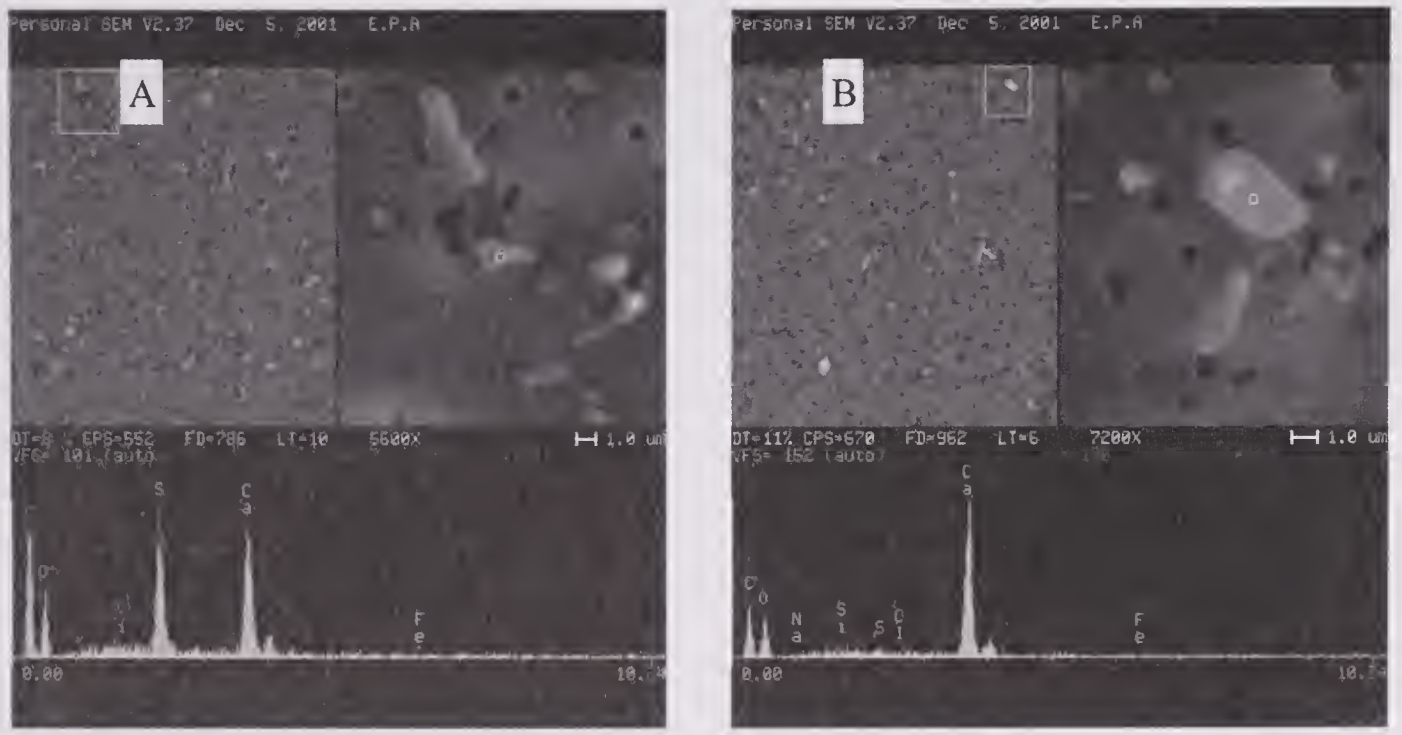


Figure 5. Particle types found in the WTC3 sample used in the nose-only inhalation exposure (Experiment B). A. Example of Ca-S particle which was prominent in the sample. B. Example of Ca particle which was prominent in the sample.

also containing Si. Some representative particles are shown in Figures 5A and 5B. In contrast to the crystals and aggregates of the bulk solid samples as described above and shown in Figure 4, the particles of WTC3 are small, typically about 1 μm, with rough, irregular features. The different form of the Ca-based particles in WTC3 reflects the dry size segregation of the inhalation exposure system. Particles with other compositions were found with far less frequency. These included particles composed of Fe, C, and Sb-Zn (one example found). One or more possible asbestos fibers (Mg-Si composition) were also found, however polarized light microscopy rather than SEM/EDX is the preferred method for identifying asbestos. SEM/EDX analysis was also performed on MSH, NIST 1649a, and ROFA and showed results typically found in previous analyses (data not shown).

6. Summary. WTC PM samples consist primarily of construction materials from the fallen-down WTC buildings. The bulk of the WTC PM samples are calcium-based compounds, specifically calcium sulfate (gypsum)

Table 6. Experiment A: Body Weights and Immediate Airway Responses. [a]

Group	B.Wt. d 0	B.Wt. d 1	Breathing Frequency (min^{-1})			PenH (unitless)		
	g	g	Pre-	Post-	% increase	Pre-	Post-	% increase
Saline	**25.8**	**24.9**	**492.3**	**348.1**	**-29.7**	**0.73**	**0.96**	**23.9**
	0.4	0.4	11.5	25.6	4.2	0.08	0.17	8.3
MSH-100	**25.5**	**25.0**	**474.0**	**320.6**	**-31.7**	**0.92**	**1.25**	**40.5**
	0.4	0.3	13.5	23.0	5.2	0.13	0.18	14.8
ROFA-10	**25.6**	**24.3**	**492.0**	**343.5**	**-29.7**	**0.74**	**1.05**	**42.3**
	0.4	0.6	14.3	18.6	4.1	0.11	0.17	13.5
ROFA-100	**25.2**	**25.2**	**461.0**	**307.0**	**-33.4**	**0.88**	**1.51**	**76.4**
	0.5	0.4	11.9	18.7	3.7	0.10	0.20	18.6
WTCX-10	**25.2**	**24.3**	**467.2**	**322.4**	**-31.1**	**0.85**	**1.14**	**52.4**
	0.6	0.5	14.7	22.5	4.1	0.15	0.18	21.8
WTCX-31.6	**25.3**	**24.7**	**476.8**	**348.5**	**-26.3**	**0.86**	**1.14**	**26.8**
	0.5	0.5	15.5	18.9	4.3	0.18	0.34	15.6
WTCX-100	**25.6**	**25.1**	**486.8**	**325.3**	**-33.1**	**0.79**	**1.11**	**40.7**
	0.6	0.4	14.1	26.6	5.1	0.08	0.16	11.8

[a] Values shown are means (in bold) and SEM immediately below means (n=12 per group). Body weight (B. Wt.) was measured in the morning. Respiratory parameters were measured immediately before (Pre-) and after (Post-) oropharyngeal aspiration of dust samples or saline on day 0. Values within solid-line boxes indicate significantly greater values in ROFA-100 mice vs. Saline mice ($P < 0.05$).

and calcium carbonate (calcite). Together these salts compose about two-thirds of WTC PM_{53} on a weight percent basis. Given the prevalent use of gypsum in ceiling tiles and wallboard, and the ease with which these building materials can be crumbled into dust, the high gypsum content is reasonable. Elemental analysis indicates that the other main components of WTC PM are construction materials such as cement and concrete aggregate. The elemental composition of WTC $PM_{2.5}$ was consistent with that of sieved unfractionated WTC PM_{53} (as WTC3, Table 5). Carbon and metal content of the WTC samples were low, as expected from crustal-derived building materials (McKetta, 1978). A more complete chemical and physical analysis of dust samples has recently been reported by the U.S. Geological Survey (USGS, 2002). In that study, dust samples were collected from undisturbed locations within a 1 km radius of the WTC site on September 17 and 18, 2001 (after the rain of September 14, 2001). The present report generally agrees with the findings of the USGS study, including the alkaline nature of the WTC PM extracts.

B. Experiment A: Dose-Response Relationships of WTC $PM_{2.5}$

1. Body weights and immediate airway responses.

Table 7. Experiment A: Diffusing Capacity of the Lung for Carbon Monoxide (DLCO) [a]

Sub-experiment:	A1	A2	A5		
Date:	11/6/01	11/8/01	12/6/01	Mean	SEM
Treatment Group				n = 3	
Saline	-3.474	-3.610	-4.510	-3.865	0.325
MSH-100	-3.734	-3.944	-4.338	-4.005	0.177
ROFA-10	-3.904	-3.597	-3.928	-3.810	0.107
ROFA-100	-4.015	-3.089	-4.293	-3.799	0.364
WTCX-10	-2.759	-3.811	-4.301	-3.624	0.455
WTCX-31.6	-4.051	-3.338	-4.014	-3.801	0.232
WTCX-100	-3.551	-4.433	-4.299	-4.094	0.275

[a] Diffusing capacity of the lung for carbon monoxide was determined one day after exposure on four mice from each treatment group, placed together in a single bell jar, in order to rapidly assess DLCO and reduce individual variability. Values shown are slopes of chamber [CO] vs. time (ppm/min), after subtraction of value from empty chamber. No significant differences among any treatment groups were detected.

Mice were exposed by oropharyngeal aspiration with $PM_{2.5}$ samples of pooled WTC sample X (10, 31.6, or 100 μg), MSH (100 μg), ROFA (10 or 100 μg), or saline on day zero. In sub-experiments A1, A2, and A5, immediate airway responses were determined on day 0, and DLCO

Table 8. Experiment A: BAL Parameters (Day 1). [a]

Group	BAL Cell Number (x 10^{-4})				Protein	LDH	Albumin	NAG
	Mac	Eos	Neut	Lym	μg/ml	U/L	μg/ml	U/L
Saline	**18.80**	**0.02**	**0.23**	**0.10**	**155.2**	**29.8**	**21.8**	**2.2**
	4.05	0.02	0.16	0.02	6.8	2.1	1.2	0.4
MSH-100	**27.78**	**0.07**	**0.78**	**0.18**	**168.8**	**27.8**	**22.3**	**2.0**
	5.96	0.03	0.21	0.05	8.8	1.7	1.2	0.4
ROFA-10	**27.36**	**0.00**	**0.21**	**0.18**	**157.9**	**32.3**	**20.8**	**3.1**
	2.70	0.00	0.05	0.07	5.3	1.2	0.9	0.4
ROFA-100	**28.69**	**0.16**	**13.18**	**0.42**	**279.5**	**93.2**	**39.2**	**7.9**
	3.98	0.06	2.44	0.09	16.8	10.3	2.8	1.2
WTCX-10	**22.28**	**0.00**	**0.09**	**0.14**	**153.7**	**30.4**	**20.8**	**1.9**
	4.03	0.00	0.02	0.04	4.3	1.8	0.8	0.3
WTCX-31.6	**31.36**	**0.01**	**0.37**	**0.21**	**160.2**	**33.6**	**21.7**	**1.8**
	7.73	0.01	0.14	0.03	6.3	1.6	1.3	0.2
WTCX-100	**20.48**	**0.06**	**1.43**	**0.23**	**161.4**	**33.7**	**21.3**	**2.3**
	1.73	0.03	0.24	0.04	4.8	2.1	1.0	0.3

[a] Values shown are means (in bold) and SEM immediately below means (n=12 per group). Bronchoalveolar lavage (BAL) cell numbers and proteins were recovered 1 day after exposure. Cell types shown are macrophages and monocytes (Mac), eosinophils (Eos), neutrophils (Neut), and lymphocytes (Lym). Total protein, lactate dehydrogenase (LDH), albumin, and N-acetyl-β-D-glucosaminidase (NAG) were measured in BAL fluid supernatant. Values within solid-line boxes indicate significantly greater values in ROFA-100 mice vs. Saline mice ($P < 0.05$). Values with dashed-line boxes indicate significantly greater values ($P < 0.05$) compared with Saline mice (excluding ROFA-100 data which generally had much larger variances than other groups).

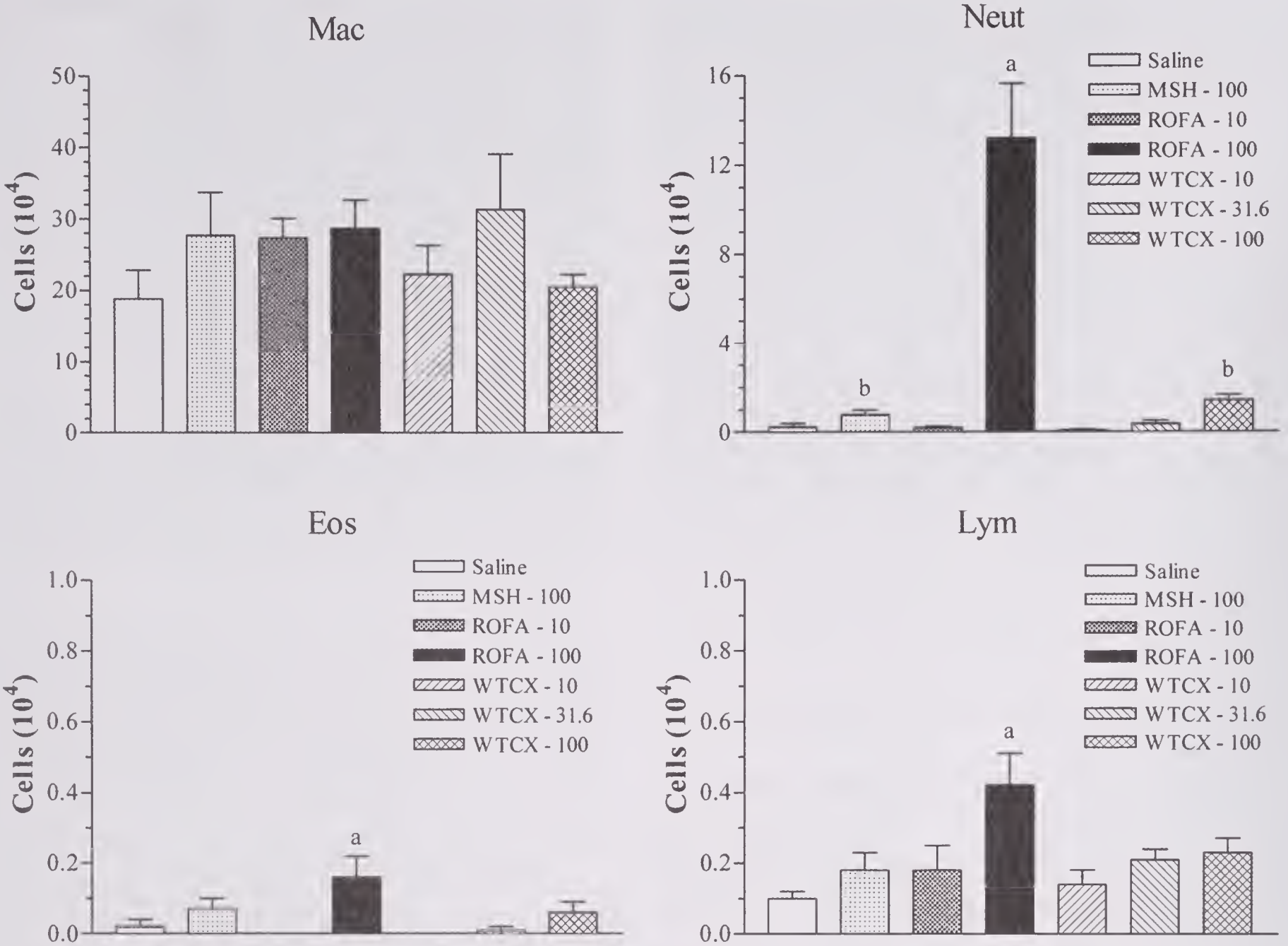


Figure 6. Experiment A. Bronchoalveolar lavage cell numbers recovered from mice one day after exposure by intratracheal instillation to PM samples in saline or saline vehicle alone. Values shown are means and SEM (n=12 per group). Cell types shown are macrophages and monocytes (Mac), eosinophils (Eos), neutrophils (Neut), and lymphocytes (Lym). [a] $P < 0.05$ vs. Saline group. [b] $P < 0.05$ vs. Saline group (comparison of rank values) after exclusion of ROFA-100 data which had much larger variances than other groups.

and BAL parameters were determined on day 1. There were no significant differences in body weights of the seven groups on day 0 or day 1 (Table 6). Ventilatory parameters were assessed in mice immediately before and after exposure. There were no differences among groups in breathing frequency, but mice exposed to the 100 μg dose of ROFA (ROFA-100) had a significant increase in PenH immediately after exposure in comparison with saline control mice (Table 6). There were no significant changes in immediate responses in mice exposed to any dose of WTCX.

*2. **DLCO.*** The diffusing capacity of the lung for carbon monoxide was determined 24 hr after oropharyngeal aspiration on groups of 4 mice from the same exposure group together in the testing chamber. There were no significant differences in DLCO among any of the groups of mice, which would be indicated by a slower uptake of CO and a reduced slope (Table 7). These data indicate that none of the PM samples caused injury severe enough to significantly reduce gas exchange at the alveolar-capillary barrier.

*3. **BAL parameters.*** Bronchoalveolar lavage parameters were determined immediately after testing for DLCO. Originally, we planned to do just two sub-experiments for this part of Experiment A. However, we noted that there was significant variation in the total cell numbers recovered from the 4 saline control mice in each of sub-experiments A1 and A2 (average of 7 x 10^4 vs. 33 x 10^4, respectively). There was no evidence of any infection (in both cases 99% of BAL cells recovered from control mice were alveolar macrophages (AMs)), mice came from the same shipment in the same week, and no

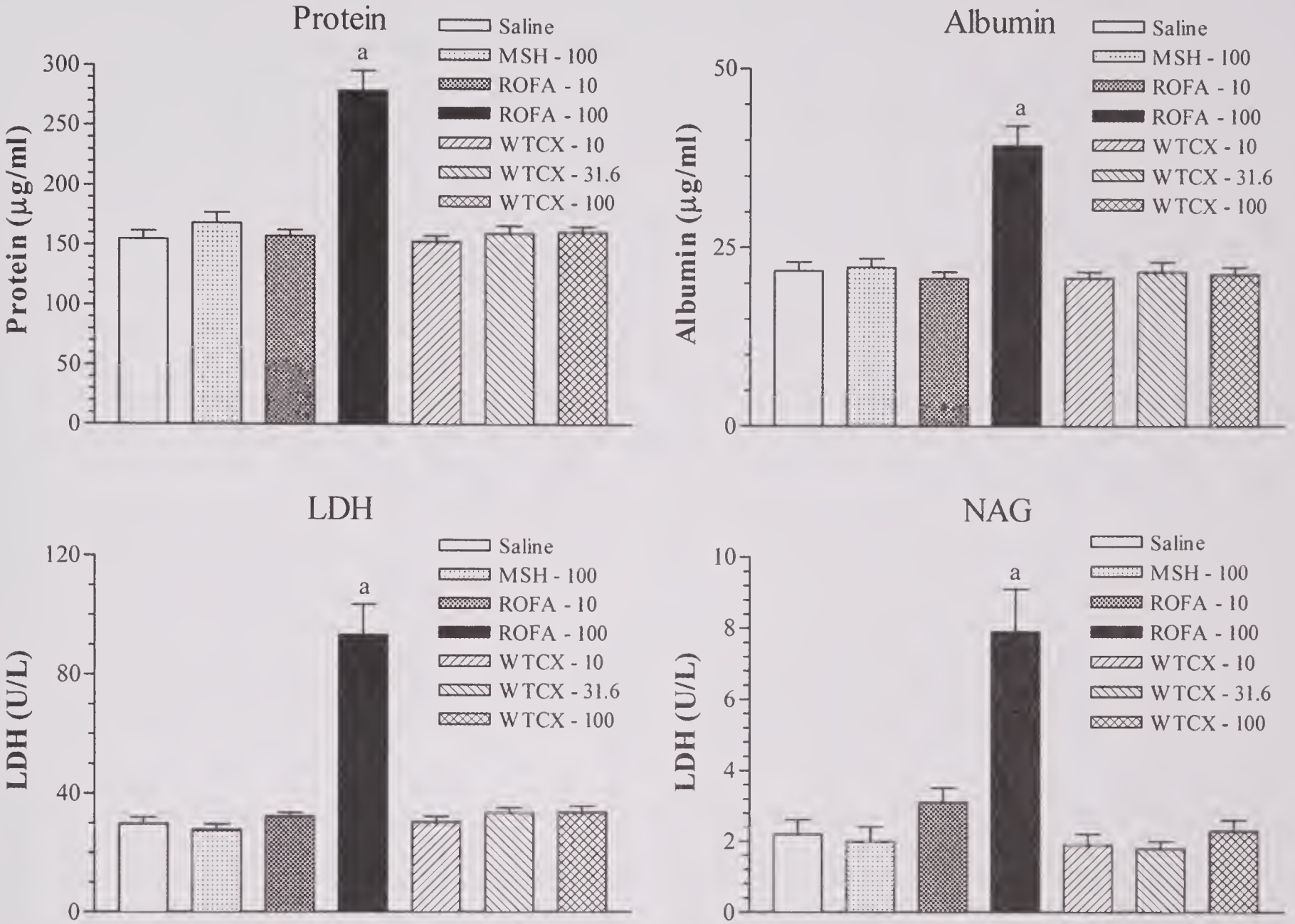


Figure 7. Experiment A. Values for total protein, lactate dehydrogenase (LDH), albumin, and N-acetyl-b-D-glucosaminidase (NAG) were measured in bronchoalveolar lavage fluid supernatants recovered from mice one day after exposure by intratracheal instillation to PM samples in saline or saline vehicle alone. Values shown are means and SEM (n=12 per group). [a] $P < 0.001$ vs. Saline group.

other reason could be deduced for the difference. Consequently, we performed a third sub-experiment to examine these endpoints and increase the number of mice per group (sub-experiment A5). The average total cell number recovered from saline control mice in A5 was 18 x 10^4 (97% AMs) - about in the middle between A1 and A2. All data shown is combined from the 3 sub-experiments. Due to the high variance of data in the ROFA-100 group, we judged that it was necessary to compare ROFA-100 data alone vs. saline control data. Other comparisons were made between the saline control group and the other groups after excluding ROFA-100 data. Significant increases in neutrophils, eosinophils, and lymphocytes were found in ROFA-100 mice compared with saline control mice (Table 8). Neutrophils comprised 31% of total BAL cells in ROFA-100 mice (Figure 6). After excluding the ROFA-100 data, significant differences in neutrophil numbers were found between the saline control group and both the MSH-100 group and the WTCX-100 group (Table 8, Figure 6; $P < 0.05$). Neutrophils comprised about 7% of total BAL cells in the WTCX-100 group, but only about 1% or less in the WTCX-31.6 and WTCX-10 groups.

Levels of proteins and enzymes were measured in the BAL supernatant to assess lung damage. Both total protein and albumin are increased after damage to the alveolar epithelial barrier (Henderson et al., 1985). Lactate dehydrogenase (LDH) is a cytoplasmic enzyme which is released by dead or dying cells, while N-acetyl-β-D-glucosaminidase (NAG) is indicative of lysosomal enzyme release (Henderson et al., 1985). All of these parameters were significantly increased in the ROFA-100 group in comparison to saline control mice (Table 8). Total protein and albumin were both increased about 80% compared to saline, while LDH was increased 3-fold and NAG almost 4-fold (Figure 7). No significant changes in BAL proteins

Table 9. Experiment A: Body Weights, Baseline PenH, and Responsiveness to Methacholine Aerosol

Group	B.Wt. d 0	B.Wt. d 1	Baseline	Dose Mch (mg/ml) and PenH AUC (PenH - sec)					
	(g)	(g)	PenH	0	4	8	16	32	64
Saline	**24.78**	**23.30**	**0.97**	**0.5**	**39.1**	**54.0**	**142.3**	**309.0**	**1249.9**
	0.42	0.65	0.13	0.5	8.4	8.9	28.5	30.6	360.7
MSH-100	**24.82**	**23.80**	**1.10**	**2.4**	**36.4**	**99.2**	**177.9**	**437.9**	**1173.3**
	0.51	0.45	0.12	0.3	5.5	22.5	23.7	54.3	358.3
ROFA-10	**24.99**	**24.55**	**0.81**	**15.5**	**42.4**	**114.6**	**205.5**	**432.4**	**1182.5**
	0.43	0.45	0.10	3.2	8.9	11.3	56.1	88.5	294.2
ROFA-100	**24.86**	**24.60**	**0.91**	**6.1**	**36.7**	**118.2**	**242.1**	**642.2**	**2190.9**
	0.44	0.45	0.11	6.3	4.1	34.1	37.2	94.0	706.4
WTCX-10	**24.96**	**24.44**	**0.92**	**18.5**	**43.9**	**88.3**	**169.1**	**281.1**	**968.4**
	0.38	0.28	0.11	6.4	11.6	16.2	52.3	35.5	129.9
WTCX-31.6	**24.56**	**23.73**	**0.85**	**15.3**	**38.3**	**55.2**	**114.4**	**249.2**	**923.3**
	0.46	0.51	0.11	3.4	4.8	8.8	7.0	39.8	174.2
WTCX-100	**24.68**	**24.14**	**1.04**	**9.5**	**67.4**	**208.3**	**397.3**	**2265.0**	**4009.1**
	0.44	0.42	0.12	8.2	11.3	47.6	61.4	260.7	580.1

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group). Body weight (B. Wt.) was measured in the morning (no significant differences were found). No significant differences were found in baseline PenH (enhanced pause; unitless) on day 1. Methacholine aerosol (Mch) was then administered (see Methods for details) at the indicated doses, and the airway response was calculated as the area under the curve (AUC) of the PenH response over time in seconds. See Figure 8 for description of statistical analysis of PenH AUC data.

and enzymes were found in any of the other PM exposure groups relative to saline controls. In general, the inflammatory response in the WTCX-100 group can be considered to be quite mild considering the fairly high dose.

4. Responsiveness to methacholine aerosol. In sub-experiments A3 and A4, the same groups of mice were exposed and the same time points were examined as described for sub-experiments A1, A2, and A5, but different endpoints were examined. There were no differences in body weights on day 0 or day 1 among the 7 groups of mice (Table 9). On day 1, there was no difference in baseline PenH values (immediately before Mch aerosol) among the 7 groups. Responsiveness to increasing concentrations of Mch aerosol was assessed and quantified by integrating the area under the PenH - time curve (PenH AUC; Table 9). In order to assess overall responsiveness and account for variability, power function equations were fit to the PenH AUC vs. [Mch] data for each group (Figure 8). The analysis showed that the Saline, MSH, ROFA-10, WTCX-10, and WTCX-31.6 groups could all be modeled with a common power function exponent (1.157). It is important to note that once the lines were determined to come from groups with a common exponent, the lines for these 5 groups were fit simultaneously, resulting in fitted equations that did not fit as well as an individual line would fit the group-specific data. The responses to saline or individual doses of Mch aerosol are not as important as the fitted line describing the groups. Among these 5 groups, ROFA-10 mice had a small but significant increase in the coefficient of the equation vs. the Saline group ($P = 0.03$). The ROFA-100 and WTCX-100 groups could be modeled with a power function with a significantly different exponent (1.471; $P = 0.001$) vs. the common exponent of the other 5 groups, indicating that these 2 groups are hyperresponsive compared with the other 5 groups. In addition, the coefficient for the WTCX-100 group was significantly different from and greater than that of the ROFA-100 group ($P = 0.0001$), showing that mice exposed to the 100 μg dose of WTCX were more reactive to Mch than the ROFA-100 group.

5. Lung histopathology. Following tests for airway responsiveness to Mch aerosol, mice from sub-experiments A3 and A4 were killed and assessed for pathological changes in the lungs. No remarkable findings were observed in the lungs of the saline control group (Table 10). In both the MSH-100 and ROFA-100 groups, focal subacute bronchiolar inflammation was found at similar incidences and average severity, which was minimal (average score: MSH-100 = 0.8; ROFA-100 = 1.0). The ROFA-10 group had a lower average severity

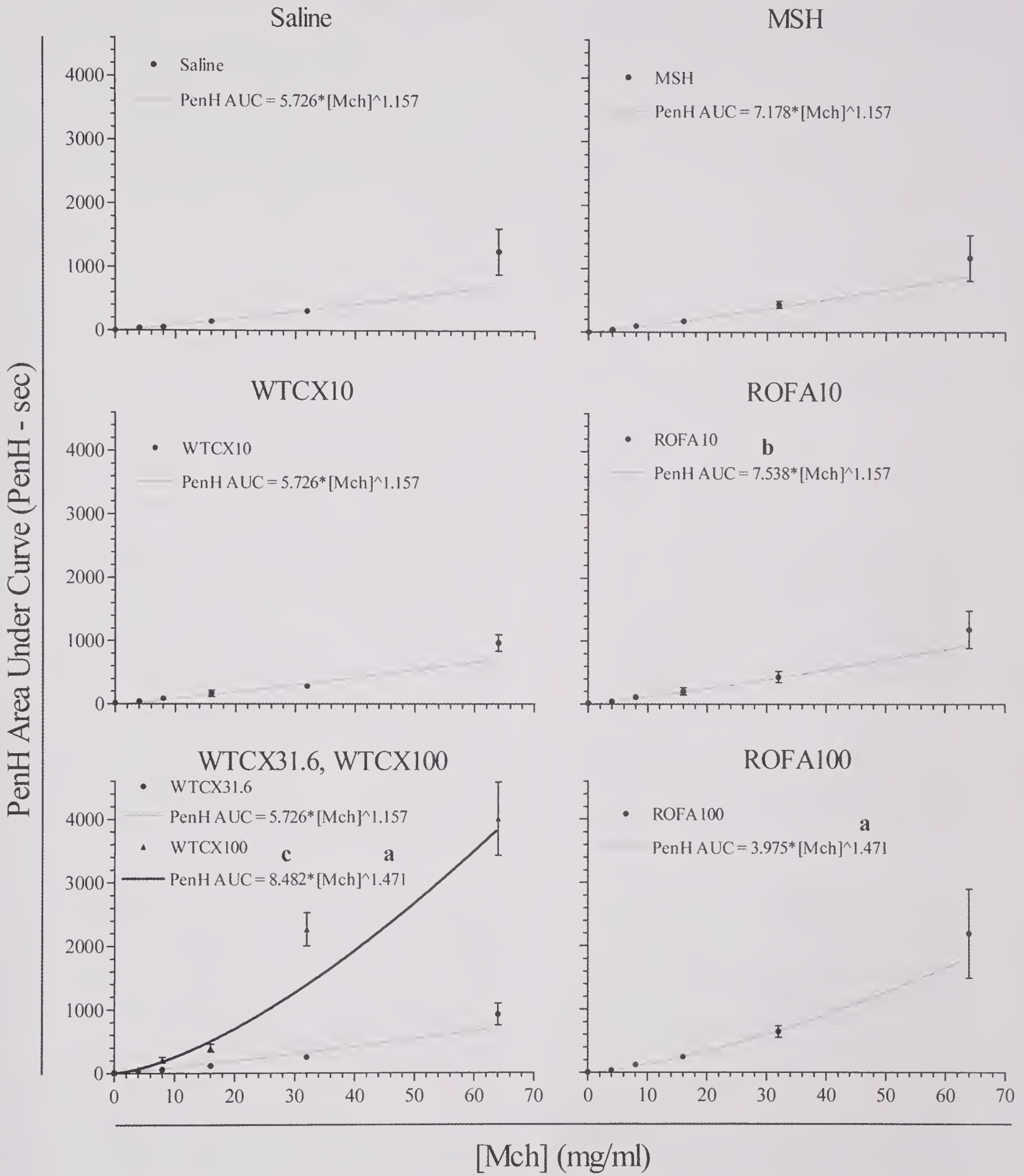


Figure 8. Experiment A: Airway responsiveness to methacholine aerosol challenge in mice exposed to PM samples or saline vehicle and tested one day later (n = 8/group; data shown are mean + SEM). Power function equations were fit to the data. Saline, MSH, ROFA10, WTCX10, and WTCX31.6 equation exponents were not significantly different. [a] Significantly different exponent vs. common Saline, MSH, ROFA10, WTCX10, and WTCX31.6 exponent ($P = 0.001$). [b] Significantly different coefficient vs. Saline coefficient ($P = 0.03$). [c] Significantly different coefficient vs. ROFA100 coefficient ($P = 0.0001$).

Table 10. Experiment A: Summary of Treatment-Related Histopathologic Findings in Mice One Day after Intratracheal Instillation of Particulate Matter Samples [a]

Treatment Group	Bronchiole, Inflammation, Subacute, Focal		Bronchiole, Pigment, Free, Focal		Bronchiole, Pigment, Macrophage, Focal		Peribronchiolar, Inflammation, Acute, Focal	
	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
Saline	0/8	0.0	0/8	0.0	0/8	0.0	0/8	0.0
MSH-100	6/8	0.8	0/8	0.0	2/8	0.3	0/8	0.0
ROFA-10	2/8	0.3	6/8	0.8	0/8	0.0	1/8	0.1
ROFA-100	6/8	1.0	8/8	1.5	0/8	0.0	0/8	0.0
WTCX-10	1/8	0.1	0/8	0.0	0/8	0.0	0/8	0.0
WTCX-31.6	0/8	0.0	1/8	0.1	0/8	0.0	0/8	0.0
WTCX-100	0/8	0.0	0/8	0.0	0/8	0.0	0/8	0.0

[a] Incidence denotes number of mice in group with finding / total number of mice examined. Average severity score for the group is shown based on the following scoring system: 0 = not present, 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high.

score (0.3) than the MSH-100 and ROFA-100 groups, and also had one mouse with minimal focal acute peribronchiolar inflammation. Although 1 mouse in the WTC-X group had a finding of minimal focal subacute bronchiolar inflammation, for an average group score of 0.1, this lesion was not found in any of the mice in the

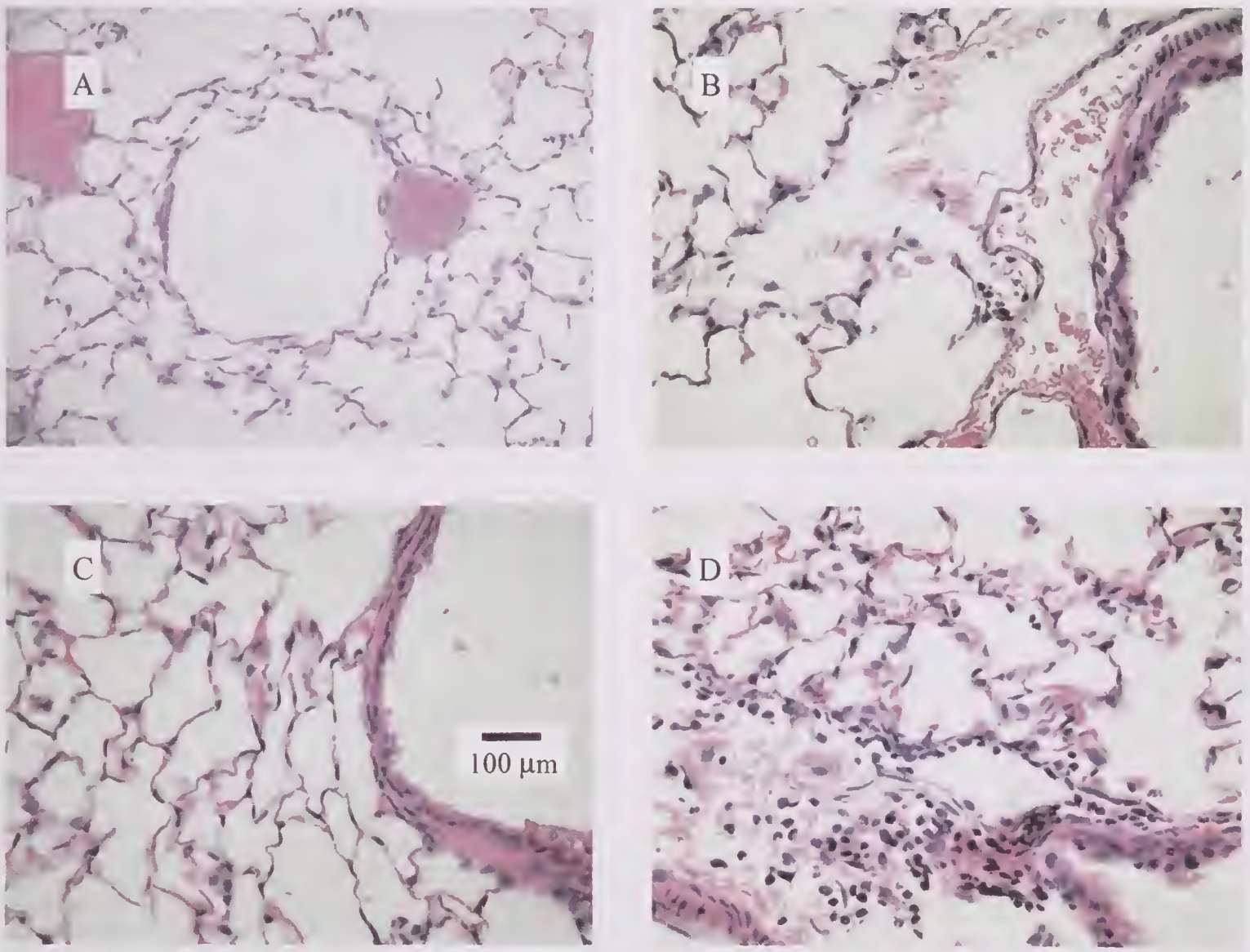


Figure 9. Experiment A: Representative micrographs of lesions occuring in lungs of mice one day after intratracheal instillation of PM samples or saline vehicle (all panels same magnification: bar length = 100 µm). A. Saline-instilled control mouse (#69) with no remarkable findings. B. Mouse #84 instilled with 100 µg MSH showing minimal degree of focal subacute bronchiolar inflammation. C. Mouse #57 instilled with 100 µg pooled WTCX sample with no remarkable findings. D. Mouse #73 instilled with 100 µg ROFA showing slight/mild degree of focal subacute bronchiolar inflammation.

WTCX-31.6 or WTCX-100 groups (Figure 9), suggesting that the lesion in the one WTCX-10 mouse was not treatment-related. Free bronchiolar pigment (presumably corresponding to PM) was identified in all ROFA-100 mice at an average severity of 1.5 (Table 10), and in 6 of 8 mice in the ROFA-10 group at an average severity of 0.8. One mouse in the WTCX-31.6 group (but none in the WTCX-100 group) had minimal free bronchiolar pigment; again suggesting that this finding is not treatment-dependent. However, it may be more difficult to see the WTC PM which is lighter in color than the ROFA or MSH PM. Focal bronchiolar macrophage pigment was found in 2 of 8 mice in the MSH-100 group at an average severity of 0.3. These findings indicate that both ROFA-100 and MSH-100, but not the pooled WTCX-100 or any lower dose, caused focal subacute bronchiolar inflammation.

*6. **Summary.*** Results from investigation of the dose-response relationships of pooled WTCX PM showed that the two lower doses of WTCX (10 μg and 31.6 μg) did not have any significant effects on inflammatory parameters, lung histopathologic findings, or respiratory responses. The 100 μg dose of WTCX caused a slight but significant increase in BAL neutrophils (7% of total cells) as determined by BAL parameters, and no inflammation as determined by histopathologic examination, while the toxic PM control, ROFA, caused minimal inflammation by histopathologic examination, significant increases in BAL neutrophils and other cell types, and significant increases in biochemical indicators of lung injury. Despite the lack of effect of WTCX on lung injury and the relatively low level of neutrophilic inflammation, mice in the WTCX-100 group were significantly more responsive to Mch aerosol challenge than all other groups. A lack of correlation between lung inflammation and airway hyperresponsiveness is not uncommon (e.g. Alvarez et al., 2000; Smith and McFadden Jr., 1995). The significant degree of airway hyperresponsiveness induced by WTC $PM_{2.5}$ implies that components of the dust can promote mechanisms of airway obstruction.

C. Experiment B: Effects of Nose-Only Inhalation Exposure

*1. **Exposure results.*** The gravimetric concentration for the WTC3 exposure chamber was 10.64 ± 3.10 mg/m^3. The mass median aerodynamic diameter (MMAD) was 1.05 μm, and the geometric standard deviation (σg) was 2.67. Chamber temperature and relative humidity was 74 °F and 11% in the control chamber and 75 °F and 11% in the WTC3 chamber. The low humidity was required to prevent the PM from sticking to the string in the aerosol generation system; the humidity within the exposure tubes was significantly higher due to body heat from the mice in a confined environment. At the end of the exposure, two control mice (#131 and #146) and one WTC3-exposed mouse (#203) were found dead in the exposure tubes, apparently from attempting to turn around in the exposure tubes and suffocating. The incidence of this problem was not unusual considering the large number of mice exposed simultaneously (AD Ledbetter, personal communication). The two spare mice (designated #146a and #203a) were used to replace the dead ones, and were killed on day 6 (December 3, 2001). An additional control mouse (designated #131a) was exposed to air for 5 hours on November 30, 2001 to replace the second dead control mouse, and was killed on day 3 (December 3, 2001). Therefore all groups of mice had the full number of 8 per group per time point.

Table 11. Experiment B: Body Weights of Mice in Nose-Only Inhalation Exposure Study [a]

	Body Weight (g)					
Day:	-1	0 (Pre-)	0.25 (Post-)	1	3	6
Group						
Air	**23.91**	**23.81**	**21.83**	**23.00**	**24.04**	**24.78**
	0.16	0.18	0.18	0.18	0.23	0.35
WTC 3	**23.93**	**23.47**	**21.82**	**23.05**	**23.89**	**24.66**
	0.15	0.18	0.16	0.18	0.15	0.34

[a] Values shown are means (in bold) and SEM immediately below means (n=48 days -1 through 1, n=32 day 3, n = 16 day 6). Body weight was measured in the morning except on day 0.25 (immediately after nose-only exposure). There was no significant difference between the two groups.

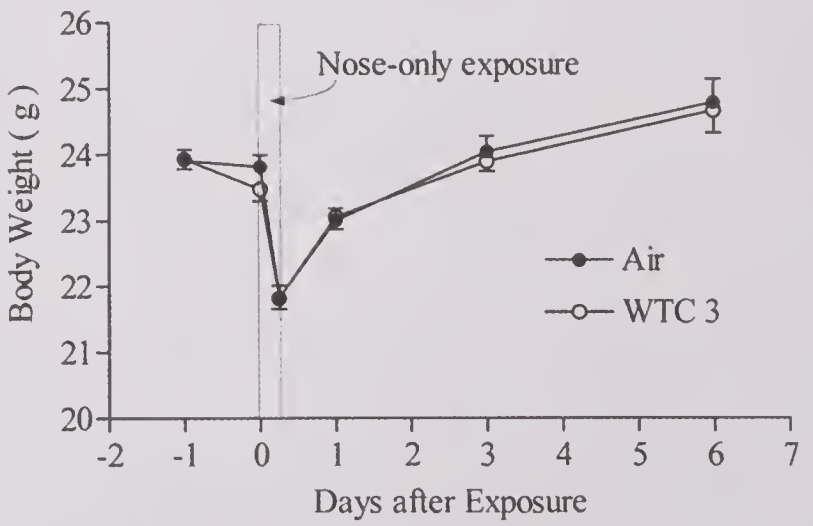


Figure 10. Experiment B: Body weights in nose-only inhalation exposure experiment. Values shown are means and SEM (numbers of mice shown in Table 11). Nose-only inhalation exposure caused a significant drop in body weight but there was no significant difference between groups.

Table 12. Experiment B: Immediate Airway Responses [a]

Group	Breathing Frequency (min^{-1})			PenH (unitless)		
	Pre-	Post-	% increase	Pre-	Post-	% increase
Air	**549.2**	**357.7**	**-35.0**	**0.88**	**1.09**	**29.5**
	9.1	24.4	4.1	0.05	0.09	13.8
WTC 3	**560.3**	**389.3**	**-30.1**	**0.94**	**1.47**	**60.1**
	10.3	19.0	3.9	0.06	0.16	18.2

[a] Values shown are means (in bold) and SEM immediately below means (n=12). Respiratory parameters were measured immediately before (Pre-) and after (Post-) nose-only inhalation exposure on day 0. No significant differences in percent change in frequency or PenH between groups were found.

*2. **Body weights.*** Animal weights were monitored on days -1 (before exposure), 0 (both before and after exposure), 1, 3, and 6 (Table 11). Body weight was measured between 7:00 and 8:00 each morning, except immediately after exposure. There were no significant differences between the two groups at any time point. The nose-only exposure caused a significant 2 g drop in body weight in both groups of mice (Figure 10).

*3. **Immediate airway responses to nose-only exposure.*** Ventilatory parameters were measured in 12 mice from each group before and after the nose-only exposure. Ventilatory rate decreased after exposure in both groups but there was no significant difference between them (Table 12). It should be noted that many physiological responses are readily reversible, and the time required to unload the mice from the exposure tubes and

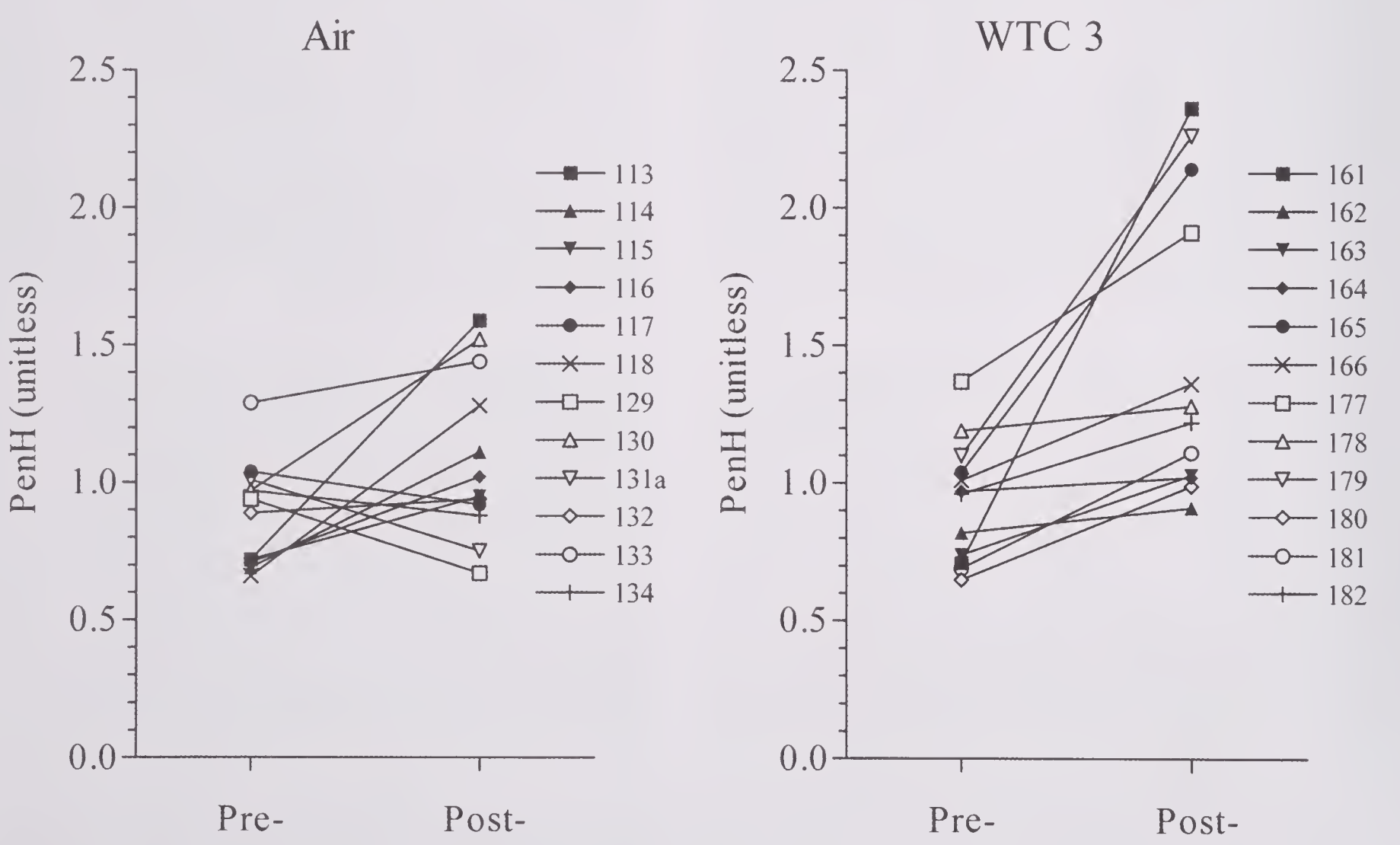


Figure 11. Experiment B. PenH values measured immediately before and after nose-only exposure to WTC 3 PM or Air only. Legends refer to individual mouse numbers. Immediate response (calculated as [(Post-value - Pre-value) / Pre-value x 100%] was not significantly different between the two groups but data indicate the possibility of individual sensitivity to dust exposure.

Table 13. Experiment B: Diffusing Capacity of the Lung for Carbon Monoxide [a]

Treatment Group	Day after Treatment	Subjects 1 - 4	Subjects 5 - 8	Average n = 2
Air	1	-3.761	-4.185	-3.973
WTC 3	1	-3.715	-3.981	-3.848
Air	3	-4.102	-3.826	-3.964
WTC 3	3	-3.818	-4.078	-3.948
Air	6	-3.528	-4.162	-3.845
WTC 3	6	-3.487	-3.809	-3.648

[a] Diffusing capacity of the lung for carbon monoxide was determined 1, 3, or 6 days after exposure on four mice from each treatment group placed together in a single bell jar. Values shown are slopes of chamber [CO] vs. time (ppm/min), after subtraction of value from empty chamber.

begin the measurement of breathing parameters (~20 minutes) may have caused us to miss some changes. PenH was increased by an average of 30% after exposure to air and by an average of 60% after exposure to WTC3 (P = 0.20). Although this difference was not significant, examination of the changes in individual mice showed that PenH increased in all 12 mice exposed to WTC3, but only 8 of 12 mice exposed to Air (Figure 11). Furthermore some of the increases in WTC3-exposed mice were quite large. These data indicate the possibility that individual mice in this outbred strain may be susceptible to bronchoconstrictive effects of WTC PM.

4. DLCO measurements. DLCO was determined 1, 3, and 6 days after exposure on 4 mice from each group placed together in the test chamber. Since there were 8 mice per group per time point, only two tests of DLCO were conducted within each group, and no statistical comparison was possible between Air and WTC3 mice. Examination of the data showed little apparent difference in DLCO at different times in the two groups (Table 13).

5. Responsiveness to methacholine aerosol. Analysis of baseline PenH values (immediately before Mch aerosol) between the two groups showed a significant difference depending on day, but not due to treatment (day 6 baselines were lower in both groups; P = 0.0007; Table 14). Responsiveness to increasing concentrations of Mch aerosol was assessed and quantified as described in the Methods section (Table 14). Unlike Experiment A, the results could be modeled with linear equations (Figure 12). Significant interactions of treatment, day, and Mch concentration were detected (P = 0.01), implying that the results depended upon a combination of factors. Slopes of the Day and Treatment combinations were significantly different (P = 0.0001). Analysis of the data showed that one equation could be used to describe the data for Air Day 6 and for WTC3 Day 1 (Figure 12). The slope of this line was significantly different from and less than that of

Table 14. Experiment B: Baseline PenH and Responsiveness to Methacholine Aerosol [a]

Treatment Group	Day after Treatment	Baseline PenH	Dose Mch (mg/ml) and PenH AUC (PenH - sec) 0	4	8	16	32	64
Air	1	**0.80**	**-1.5**	**20.0**	**61.5**	**262.0**	**945.9**	**1940.7**
		0.04	10.8	15.3	41.7	71.4	227.1	597.3
WTC 3	1	**0.68**	**21.8**	**18.8**	**88.9**	**227.2**	**697.8**	**492.3**
		0.05	7.9	20.8	12.7	50.0	175.4	104.0
Air	3	**0.76**	**-13.7**	**50.4**	**238.4**	**384.9**	**1188.1**	**2031.4**
		0.05	9.8	20.9	68.7	77.4	172.5	178.8
WTC 3	3	**0.86**	**-11.4**	**15.2**	**37.8**	**198.6**	**679.1**	**1293.3**
		0.07	9.7	8.3	12.6	108.8	317.2	356.6
Air	6	**0.61**	**-3.5**	**-7.6**	**35.5**	**106.5**	**70.1**	**786.6**
		0.05	3.8	12.4	11.7	43.6	59.5	269.9
WTC 3	6	**0.58**	**2.0**	**31.1**	**72.0**	**276.7**	**1030.4**	**1935.6**
		0.04	2.9	9.0	20.4	97.1	252.8	385.1

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group). Baseline PenH (enhanced pause; unitless) was measured immediately before methacholine aerosol challenge. No significant differences were found between treatment groups, but there was a significant difference in day, with day 6 values being significantly lower than other days (solid line box; P = 0.0007). Methacholine aerosol (Mch) was administered (see Methods for details) at the indicated doses, and the airway response was calculated as the area under the curve (AUC) of the PenH response over time in seconds. See Figure 12 for description of statistical analysis of PenH AUC data.

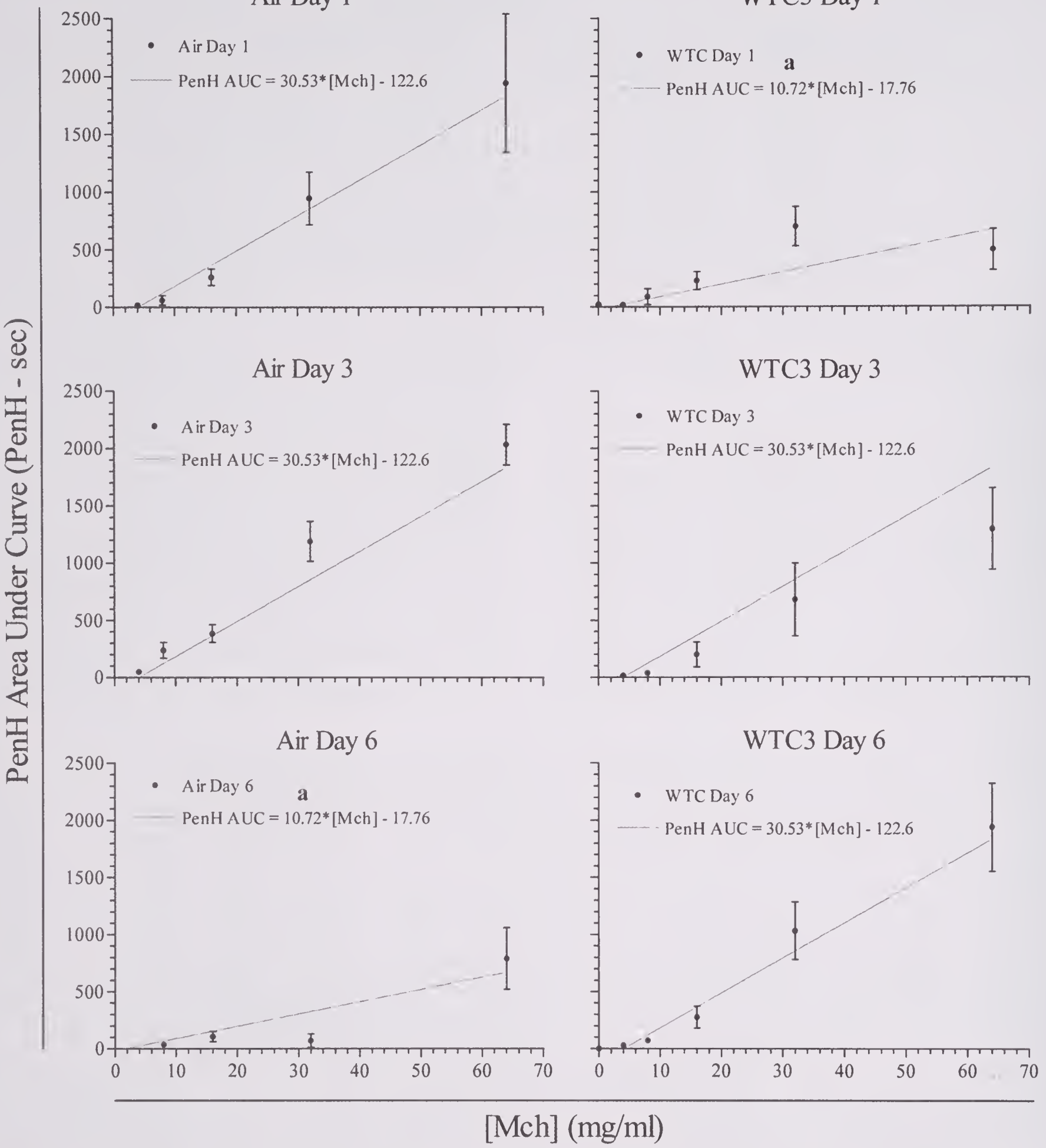


Figure 12. Experiment B: Airway responsiveness to methacholine aerosol challenge in mice exposed nose-only to Air or aerosolized WTC (sample 3) and tested 1, 3, or 6 days later (n = 8/group; data shown are mean + SEM). Linear dose-response relationships were found. [a] Slope of Air Day 6 and WTC3 Day 1 were significantly different from and lower than the 4 other groups.

the equation used to fit the other four groups. It should be noted that as in Experiment A, once the lines were determined to come from groups with equal slopes, the lines were fit simultaneously. This resulted in an equation for the common groups (e.g. Air Day 6 and WTC3 Day 1) that did not fit as well as lines fit to the individual group

Table 15. Experiment B: BAL Cell Numbers after Nose-Only Exposure [a]

Group	Day	BAL Cell Number (x 10^{-4})			
		Mac	Neut	Eos	Lym
Air	1	**14.80**	**0.012**	**0.003**	**0.046**
		3.11	0.004	0.002	0.010
WTC 3	1	**17.48**	**0.006**	**0.000**	**0.067**
		3.13	0.003	0.000	0.013
Air	3	**16.56**	**0.008**	**0.000**	**0.125**
		1.13	0.004	0.000	0.041
WTC 3	3	**26.72**	**0.034**	**0.016**	**0.197**
		3.11	0.017	0.009	0.036
Air	6	**22.24**	**0.000**	**0.000**	**0.140**
		1.13	0.000	0.000	0.026
WTC 3	6	**29.86**	**0.019**	**0.005**	**0.281**
		2.58	0.008	0.004	0.056

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group). Solid-line box: Significant difference ($P = 0.01$) between Air and WTC3, Day 6 different from Day 1. Dashed-line boxes: Significant difference ($P = 0.02$) between Air and WTC3, Day 3 and Day 6 both different from Day 1.

data. These results could be interpreted as saying that mice exposed to WTC3 became more responsive to Mch in the days following exposure, while Air-exposed mice became less responsive. However, close examination of the data from experiment B showed it was more variable than that from experiment A. Therefore, although the Air Day 6 and WTC3 Day 1 groups were less responsive to Mch aerosol challenge than the other four groups, the biological significance of this finding is unclear.

6. BAL parameters. Numbers of BAL cells were quantified 1, 3, and 6 days after nose-only exposure to Air or WTC3 (Table 15). Analysis of the data showed that mice exposed to WTC3 had significantly greater numbers of macrophages ($P = 0.01$) and lymphocytes ($P = 0.02$) compared with Air-exposed mice. Macrophage numbers were significantly greater on Day 6 vs. Day 1, and lymphocyte numbers were significantly greater on both Day 3 and Day 6 vs. Day 1. Over all time points, WTC3 mice had 38% more macrophages, and 75% more lymphocytes (Figure 13). However, macrophages comprised 99% of all recovered cells in both groups at all time points. Lymphocytes constituted about 1% or less of total BAL cells, while both

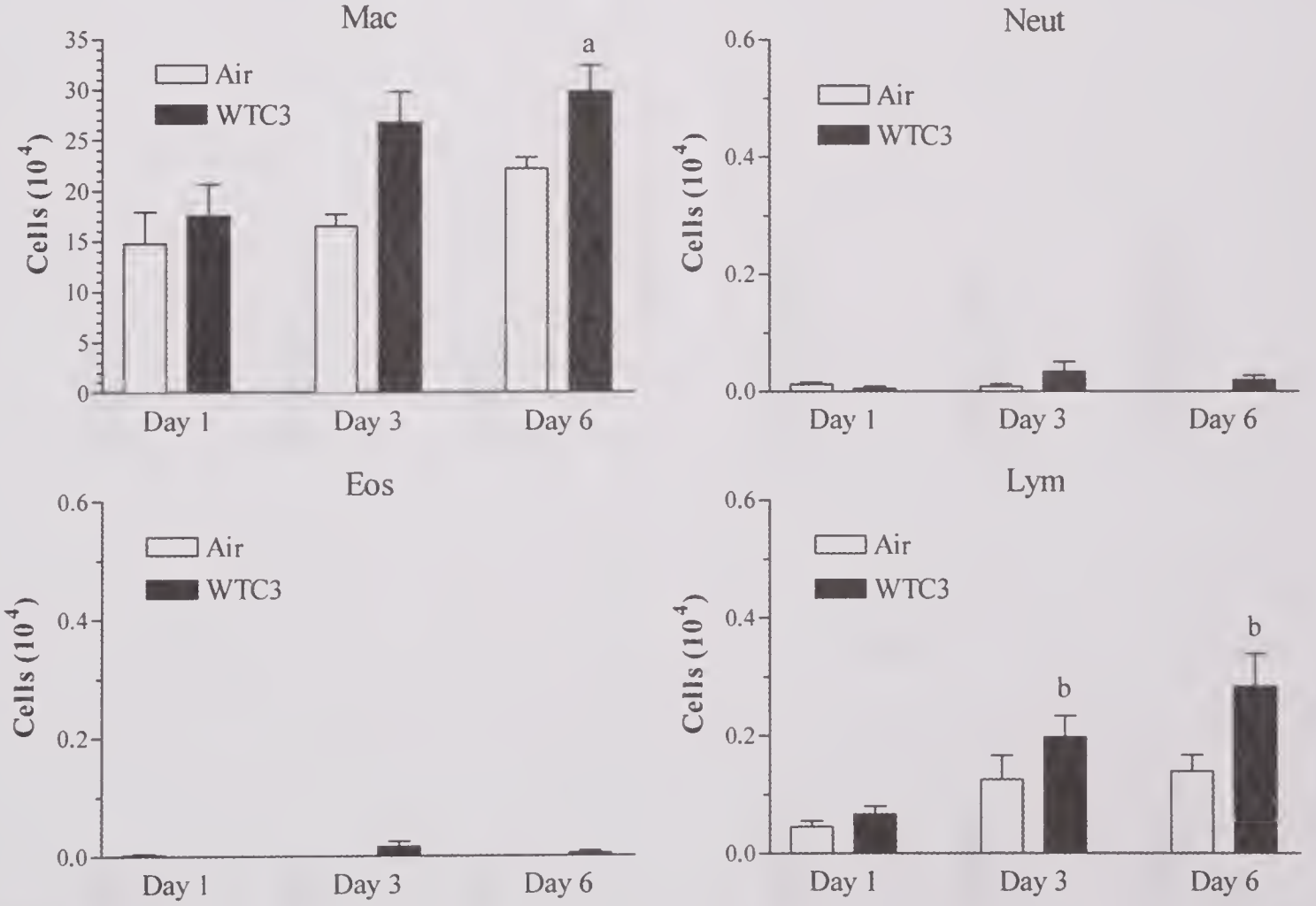


Figure 13. Experiment B: Bronchoalveolar lavage cell numbers recovered from mice 1, 3, or 6 days after 5 hr nose-only inhalation exposure to WTC sample 3 or Air only. Values shown are means and SEM (n=8 per group). Cell types shown are macrophages and monocytes (Mac), eosinophils (Eos), neutrophils (Neut), and lymphocytes (Lym). [a] Significant difference ($P = 0.01$) between Air and WTC3, Day 6 different from Day 1. [b] Significant difference ($P = 0.02$) between Air and WTC3, Day 3 and Day 6 different from Day 1.

Table 16. Experiment B: BAL Supernatant Biochemical Values after Nose-Only Exposure [a]

Group	Day	Protein µg/ml	Albumin µg/ml	LDH U/L	NAG U/L
Air	1	**165.2**	**21.0**	**29.0**	**1.5**
		6.1	1.1	3.4	0.1
WTC 3	1	**147.1**	**16.9**	**23.9**	**1.6**
		6.7	1.2	3.3	0.1
Air	3	**136.6**	**16.2**	**33.0**	**1.8**
		10.4	1.2	6.4	0.0
WTC 3	3	**138.1**	**15.8**	**28.9**	**1.6**
		7.8	1.5	3.4	0.1
Air	6	**172.6**	**22.4**	**30.2**	**1.4**
		8.5	1.3	2.4	0.2
WTC 3	6	**146.5**	**17.5**	**27.1**	**1.4**
		6.8	1.1	3.1	0.1

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group). Heavy solid-line boxes: Significant overall treatment effect (WTC3 < Air; no significant day effect); $P = 0.05$ (Protein) or $P = 0.007$ (Albumin).

neutrophils and eosinophils were about 0.1% or less of total BAL cells, indicating that WTC3 did not induce a significant acute inflammatory reaction. The increase in macrophages and lymphocytes is probably a nonspecific reaction to inhalation of large amounts of dust which induces macrophage recruitment for phagocytosis and clearance of the particles (Adamson and Bowden, 1981).

Levels of proteins and enzymes in the BAL supernatant were assessed in the two groups of mice at 3 time points (Table 16). Significant differences between Air and WTC3 groups were found for total protein ($P = 0.05$) and albumin ($P = 0.007$), but there was no significant day effect. Surprisingly, the levels of protein and albumin were higher in the Air group. However, the overall levels of all proteins and enzymes was low in both groups and at all time points (Figure 14), in comparison with Experiment A. The results indicate that at this exposure concentration and duration, WTC3 $PM_{2.5}$ does not induce severe acute lung injury.

*7. **Nasal histopathology.*** No exposure-related nasal lesions were found in mice exposed to air alone (controls). Similarly no nasal lesions were found in the mice exposed to WTC3 and killed 3 or 6 days post-exposure. None of the mice in any group had

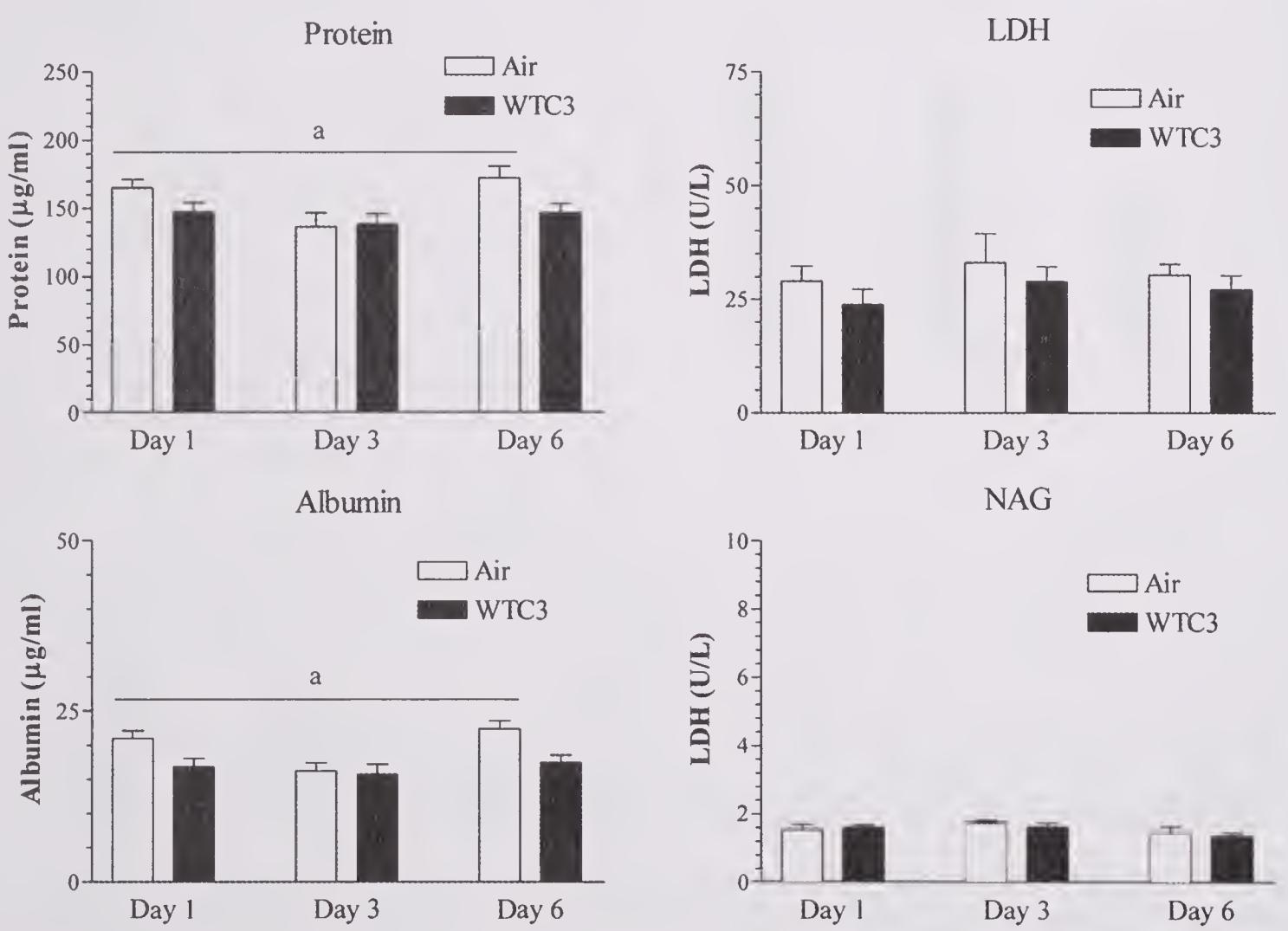


Figure 14. Experiment B: BAL supernatant biochemical values in mice 1, 3, or 6 days after 5 hr nose-only inhalation exposure to WTC3 or Air only. Values shown are means and SEM (n=8 per group). [a] Significant overall treatment effect (WTC3 < Air; no significant day effect); $P = 0.05$ (Protein) or $P = 0.007$ (Albumin).

exposure-related alterations in the mucosal tissues lined by respiratory or olfactory epithelium in the more distal tissue sections examined (T2 and T3).

The only nasal alterations observed by light microscopic examination was minimal to mild acute, focal inflammation (rhinitis) in four of the eight mice exposed to WTC3 $PM_{2.5}$ and killed 24 h post-exposure (animal # 161, 162, 163, 164). This minimal inflammatory response was bilateral and restricted to the most proximal tissue section examined (T1). It was characterized by a slight increase in the number of neutrophils in the mucosal tissues lining the lateral meatus, especially in the ventral lateral meatus, the dorsomedial aspect of the proximal maxilloturbinate, and the ventral aspect of the proximal nasoturbinate in both nasal passages. It must be emphasized, however, that the severity of this focal rhinitis was minimal to mild (i.e., severity score of 1 or 2 out of 4). In addition, there were no associated histologic alterations in the surface epithelium or in the subepithelial tissues in the affected areas. In mouse # 162 there was a small accumulation of mucus and fiber-like material in the lateral meatus of one nasal passage in T1.

In summary, some but not all mice exposed to WTC3 and killed 1 day after exposure had a minimal acute rhinitis that was restricted to the proximal nasal airways. This minimal inflammatory response was probably due to stimulation by the WTC3 exposure. This stimulation, however, did not result in any apparent epithelial cell injury that is often observed with many inhaled agents. No nasal lesions were observed in mice exposed to WTC3 and killed 3 or 6 days post-exposure. This suggests that any acute inflammation that may have been induced by the dust exposure quickly resolved and did not result in any persistent injury to the nasal mucosa that could be detected by light microscopy.

8. Lung histopathology. No remarkable findings were observed in any of the mice exposed to Air or to WTC3 at any time point. Since nasal lesions as described above were restricted to the proximal T1 region and were not found in the more distal T2 and T3 regions, the lack of any findings in the lung suggests that the proximal region of the nose effectively scrubbed out enough of the particulate matter during the exposure to WTC3 to limit deposition further down the respiratory tract. It should be noted that mice are obligate nose-breathers, while humans have significant oral breathing, and therefore significantly more PM can bypass the nasal passages in humans (Schlesinger, 1985). Studies have shown considerably less deposition efficiency in the alveolar region of rodents compared with humans (Asgharian et al., 1995).

9. Summary. Results from investigation of the effects of nose-only exposure to the WTC3 sample indicate that WTC3 $PM_{2.5}$ induced mild transitory neutrophilic inflammation in proximal nasal airways of some mice, but WTC3 $PM_{2.5}$ did not induce neutrophilic inflammation in the lungs of any mice. However, numbers of macrophages were significantly increased after exposure, suggesting that some WTC3 $PM_{2.5}$ penetrated into the lower respiratory tract, which stimulated recruitment of macrophages to phagocytize and clear the particulate matter. Biochemical parameters of lung injury were not increased at all by WTC3. The data suggested that individual mice in this outbred strain may be sensitive to the immediate effects of WTC3 exposure and respond with increased airway obstruction, although this effect was not significant for the group as a whole. Groups of mice exposed to Air or WTC3 $PM_{2.5}$ differed in their responsiveness to Mch aerosol at different times after exposure, but the biological significance of these results was unclear. The dose deposited in the respiratory tract following nose-only inhalation may be estimated as follows: 18.8 ml/min (mouse minute ventilation based on weight; Costa et al., 1992) x 300 min (exposure time) x 0.001 L/ml x 0.001 m^3/L x 10.64 mg/m^3 (exposure concentration) x 1000 μg/mg x 0.23 (deposition efficiency estimate in total respiratory tract) ≈ 14 μg. Thus, the significant difference in dose deposited into the airways between oropharyngeal aspiration (100 μg) and nose-only inhalation probably accounts for the lack of effect in many of the endpoints examined following nose-only inhalation exposure.

D. Experiment C: Effect of Geographical Location of WTC PM Samples on Responses

1. Sub-experiments and body weights. WTC $PM_{2.5}$ samples from 7 different sites comprised the pooled WTCX sample (Figure 1, Table 1). The effects of the pooled sample may have been dominated by one or more site samples which were toxic in comparison with other site samples. Experiment C was designed to address this possibility and to examine the variability of pulmonary responses associated with WTC $PM_{2.5}$ samples collected from different geographical locations. The 7 sites were located east (WTC11 - 0.1 miles, WTC8 - 0.4 miles), southeast (WTC13 - 0.1 miles, WTCF - 0.25 miles), south (WTCB - 0.25 miles), west-northwest (WTCC - 0.2 miles), and north-northeast (WTCE - 0.25 miles) from the center point of Ground Zero. Sub-experiment C1 examined responses to WTC8, WTC13, WTCF, NIST, and Saline control mice. Sub-experiment C2 examined responses to WTC11, WTCB, WTCC, WTCE, and Saline control mice.

Table 17. Experiment C: Body Weights [a]

Group	Sub-Experiment	Body Weight (g) Day -1	Day 0	Day 1	Day 3
Saline	C1	**24.69**	**24.76**	**24.72**	**24.91**
		0.39	0.42	0.45	0.42
WTC8	C1	**24.76**	**24.54**	**24.16**	**25.35**
		0.49	0.52	0.58	0.90
WTC13	C1	**24.69**	**24.26**	**23.91**	**24.14**
		0.40	0.42	0.38	0.43
WTCF	C1	**24.72**	**24.46**	**24.17**	**24.41**
		0.43	0.42	0.38	0.49
NIST	C1	**24.75**	**24.32**	**24.15**	**24.31**
		0.50	0.53	0.50	0.71
Saline	C2	nd[b]	**22.55**	**22.23**	**22.85**
			0.27	0.25	0.55
WTC11	C2	nd	**23.72**	**23.20**	**23.72**
			0.39	0.43	0.68
WTCB	C2	nd	**23.66**	**23.11**	**23.29**
			0.33	0.33	0.61
WTCC	C2	nd	**23.67**	**23.12**	**23.96**
			0.27	0.24	0.41
WTCE	C2	nd	**23.69**	**23.27**	**23.44**
			0.29	0.36	0.41

[a] Values shown are means (in bold) and SEM immediately below means (on days -1, 0, and 1, n=16 per group, except Saline sub-experiment C2: n=8; on day 3, n=8 per group, except Saline sub-experiment C2: n=4). [b] nd - Not determined. No treatment-related differences in body weight among groups within each sub-experiment were detected.

Responses were examined in 8 mice per group at 1 and 3 days after oropharyngeal aspiration of 100 μg of each PM sample or saline alone (n = 4 per time point in sub-experiment C2 Saline mice). Responses were examined at both 1 and 3 day time points in order to begin examination of persistence of exposure effects. Statistical analysis of the data was performed within each sub-experiment.

Body weights were determined on days -1 (before oropharyngeal aspiration), 0, 1, and 3 in sub-experiment C1, and on days 0, 1, and 3 in sub-experiment C2 (Table 17). No treatment-related differences in body weight among groups within each sub-experiment were detected, although there were differences on the day the animals were weighed ($P = 0.0001$).

2. Responsiveness to methacholine aerosol. In sub-experiment C1, the WTC8 group had significantly greater baseline PenH values 1 day after exposure compared with the WTC13 group (Table 18). No other significant differences in baseline PenH values in sub-experiments C1 or C2 were found. Responsiveness to methacholine aerosol was quantified as PenH AUC (Table 18). Analysis of the data in both sub-experiments showed that linear regression equations could be fit to the PenH AUC vs. [Mch] data (Figure 15). In both sub-experiments, tests for equal slopes on days 1 and 3 after exposure showed that day was not a significant factor. Therefore, a single equation was fit to the data for each group, and day does not appear in the equations.

In sub-experiment C1, the WTC8, WTCF, and NIST groups could be described with a common slope and intercept. The common slope of these 3 groups was significantly different from and greater than that of the WTC13 or Saline C1 groups ($P < 0.0005$), indicating that WTC8, WTCF, and NIST were hyperresponsive to methacholine aerosol. The slope of the WTC13 group was significantly greater than that of the Saline C1 group, showing that WTC13 mice were hyperresponsive compared with control mice, though less so than WTC8,

Table 18. Experiment C: Baseline PenH and Responsiveness to Methacholine Aerosol [a]

Group	Sub-Experiment	Day	Baseline PenH	Dose Mch (mg/ml) and PenH AUC (PenH - sec) 0	4	8	16	32	64
WTC8	C1	1	**0.68**	**35.2**	**69.6**	**197.0**	**429.4**	**1115.0**	**1953.5**
			0.05	10.1	19.2	92.8	87.0	126.6	203.5
WTC13	C1	1	**0.45**	**35.5**	**50.5**	**87.9**	**264.7**	**674.8**	**1151.3**
			0.02	5.1	4.4	9.1	44.2	135.4	206.6
WTCF	C1	1	**0.55**	**25.9**	**62.3**	**164.4**	**369.1**	**1145.3**	**1966.5**
			0.05	2.1	14.4	29.0	47.3	155.8	293.0
NIST	C1	1	**0.59**	**31.6**	**52.7**	**97.3**	**279.3**	**811.1**	**1882.2**
			0.03	3.1	2.9	11.5	54.0	149.2	324.6
Saline	C1	1	**0.57**	**24.2**	**40.4**	**61.4**	**171.7**	**534.5**	**1077.0**
			0.02	1.5	4.0	6.6	19.9	74.2	163.2
WTC8	C1	3	**0.66**	**14.5**	**41.9**	**62.2**	**182.5**	**690.3**	**1907.0**
			0.04	3.0	10.9	25.1	49.7	206.4	370.0
WTC13	C1	3	**0.78**	**27.9**	**51.2**	**207.0**	**271.9**	**1000.8**	**1554.5**
			0.08	9.0	9.7	62.6	68.3	204.3	190.8
WTCF	C1	3	**0.77**	**18.8**	**29.3**	**87.5**	**280.8**	**994.4**	**2160.8**
			0.07	6.4	22.3	34.4	130.7	368.1	563.2
NIST	C1	3	**0.76**	**21.1**	**47.1**	**86.1**	**212.3**	**962.5**	**1886.2**
			0.05	8.0	14.8	19.4	72.2	271.0	437.2
Saline	C1	3	**0.71**	**-2.8**	**30.6**	**99.2**	**280.4**	**481.0**	**1287.9**
			0.03	4.6	17.2	16.8	67.0	40.8	79.5
WTC11	C2	1	**0.69**	**15.9**	**66.9**	**238.8**	**811.0**	**1496.5**	**1835.9**
			0.06	6.5	23.3	90.7	223.6	437.7	306.0
WTCB	C2	1	**0.67**	**7.3**	**46.4**	**205.4**	**695.5**	**1561.4**	**2333.1**
			0.05	9.4	10.0	53.8	193.0	404.0	611.8
WTCC	C2	1	**0.62**	**5.9**	**27.5**	**118.7**	**737.6**	**1826.9**	**2800.2**
			0.06	5.9	9.9	30.6	249.0	466.3	672.8
WTCE	C2	1	**0.62**	**8.3**	**28.7**	**155.6**	**390.0**	**1112.5**	**1722.8**
			0.02	3.1	12.6	86.8	177.1	360.2	452.6
Saline	C2	1	**0.61**	**3.2**	**52.5**	**111.7**	**167.7**	**308.7**	**825.5**
			0.08	4.0	7.5	19.3	29.5	26.0	101.6
WTC11	C2	3	**0.59**	**3.8**	**24.7**	**45.0**	**256.8**	**1003.0**	**2304.1**
			0.05	6.3	12.5	19.2	112.0	325.5	559.8
WTCB	C2	3	**0.59**	**22.2**	**31.9**	**98.0**	**238.6**	**738.5**	**2609.2**
			0.05	4.3	14.6	34.7	59.2	216.6	585.5
WTCC	C2	3	**0.56**	**10.0**	**22.8**	**118.9**	**351.7**	**1282.4**	**2669.0**
			0.04	7.1	6.6	37.4	144.1	365.5	520.9
WTCE	C2	3	**0.62**	**5.7**	**42.3**	**138.6**	**412.4**	**1257.9**	**2088.7**
			0.05	6.3	11.7	33.2	117.6	297.5	472.9
Saline	C2	3	**0.62**	**3.5**	**27.5**	**84.0**	**114.7**	**341.1**	**1058.7**
			0.05	3.5	27.0	65.1	17.2	65.2	45.7

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group, except Saline sub-experiment C2: n=4). A significant difference in baseline PenH (enhanced pause; unitless) was found between the WTC8 group and the WTC13 group on day 1 (heavy solid line box). No other significant differences in baseline values on day 1 or day 3 were detected. Methacholine aerosol (Mch) was administered at the indicated doses, and the airway response was calculated as the area under the curve (AUC) of the PenH response over time in seconds. See Figure 15 for description of statistical analysis of PenH AUC data.

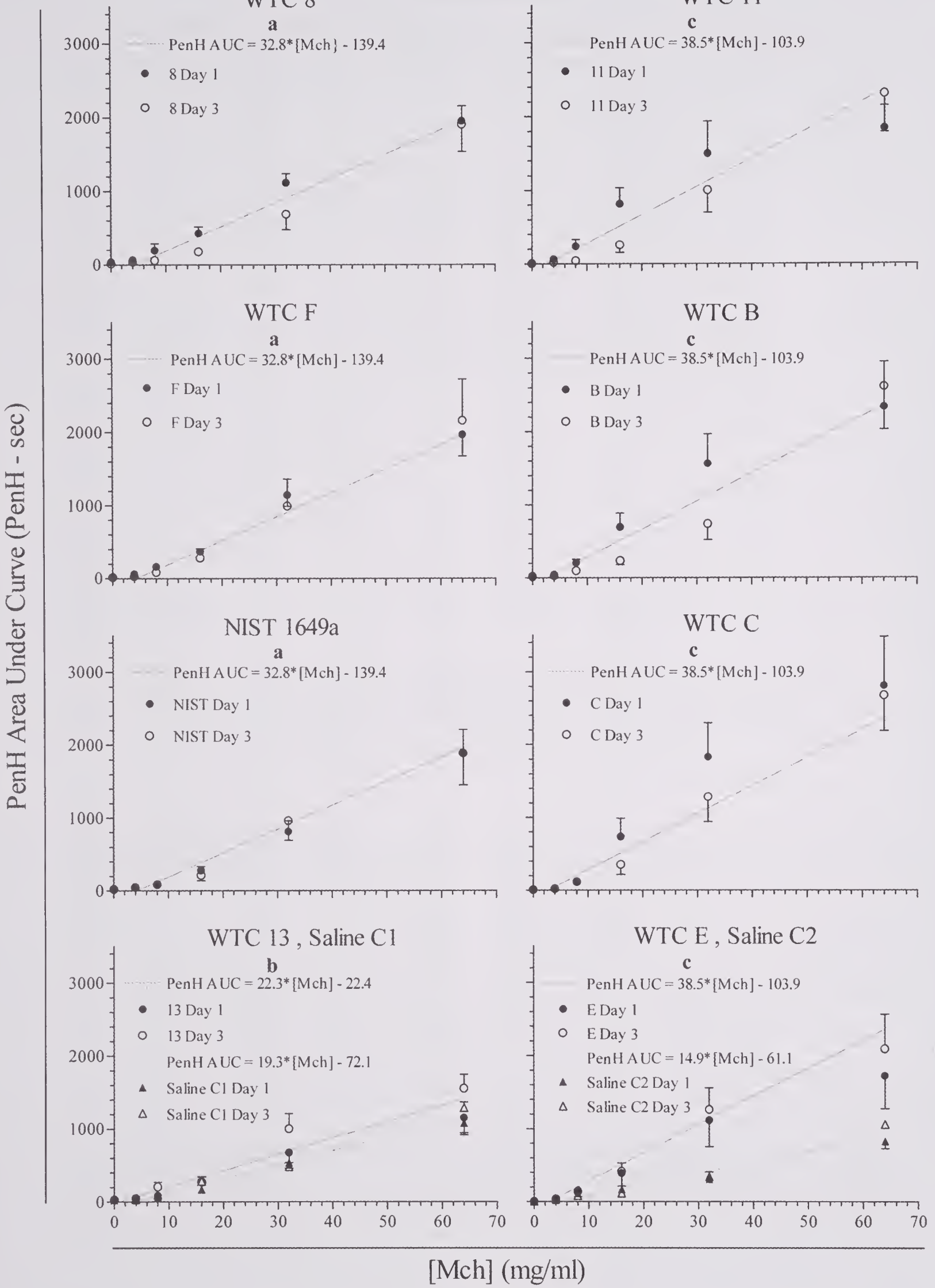


Figure 15. Experiment C: Airway responsiveness. See next page for figure legend.

Figure 15. (previous page.) Experiment C: Airway responsiveness to methacholine aerosol challenge in mice exposed to saline vehicle, NIST 1649a, or WTC PM samples from individual collection sites and tested 1 or 3 days later (n = 8 per group except Saline sub-experiment C2: n = 4). A single regression equation was fit to the data for both days in each group. [a] In sub-experiment C1 (left panels), a common equation could be fit to the WTC8, WTCF, and NIST data, and the slope of the line was significantly different from and greater than the slopes of the WTC13 and Saline C1 equations. [b] In sub-experiment C1 (left panels), the slope of the equation for the WTC13 group was significantly different from and greater than the slope for the Saline C1 group. [c] In sub-experiment C2 (right panels), a common equation could be fit to the WTC11, WTCB, WTCC, and WTCE data, and the slope of the line was significantly different from and greater than the slope of the Saline C2 equation.

WTCF, and NIST mice.

In sub-experiment C2, the WTC11, WTCB, WTCC, and WTCE groups could all be described with a common slope and intercept, which was similar to that found for WTC8, WTCF, and NIST groups in sub-experiment C1. The common slope of the 4 WTC groups was significantly different from and greater than that of the Saline C2 group ($P = 0.001$), indicating that WTC11, WTCB, WTCC, and WTCE were hyperresponsive to methacholine aerosol.

In general, these results are consistent with those from Experiment A, where the 100 μg dose of the pooled WTCX sample induced significant hyperresponsiveness to methacholine aerosol compared with control PM samples and saline. All but one of the WTC PM samples, as well as the NIST control PM, appeared to cause similar degrees of hyperresponsiveness. However, the WTC13 sample, located just 0.1 miles southeast of Ground Zero, caused a lower degree of hyperresponsiveness compared with WTC8, WTCF, and NIST.

3. BAL cells. After assessment of responsiveness to Mch aerosol, mice were killed and numbers of BAL cells were quantified (Table 19; Figure 16). In sub-experiment C1, significant increases in numbers of neutrophils on Day 1 were found in all PM-exposed groups compared with Saline C1 mice. An average of 14.7×10^4 neutrophils was recovered from NIST mice (45% of total BAL cells). Significantly lower numbers of neutrophils were found in WTC13 (6.1×10^4) and WTCF (6.9×10^4) mice, while numbers of neutrophils were lower still in WTC8 mice (3.2×10^4). The neutrophilic response abated by Day 3, and there were no significant differences among the 5 groups. Numbers of lymphocytes were significantly increased in WTC8, WTC13, WTCF, and NIST mice in comparison with Saline C1 mice on both Day 1 and Day 3 after oropharyngeal aspiration ($P = 0.0001$). Lymphocyte numbers significantly increased in all groups from Day 1 to Day 3 ($P = 0.0001$). Since there were significant interactions between day and treatment with respect to eosinophil numbers ($P = 0.01$), no significant differences among groups could be discerned. Although a significant difference in macrophage numbers was detected in the WTC13 group compared with saline, it was very marginal and not considered biologically significant.

In sub-experiment C2, significant increases in neutrophils and eosinophils were found in WTC11 and WTCE mice compared with Saline C2 mice. The average number of neutrophils in these 2 WTC groups was comparable to those found in the WTC13 and WTCF groups in sub-experiment C1. In addition, numbers of neutrophils and eosinophils were significantly greater in WTCE mice compared with WTCB mice. Neutrophils numbers declined from Day 1 to Day 3 ($P = 0.0001$). It should be noted that of the four mice in the Saline C2 Day 1 group, two had unusually high neutrophil numbers (individual numbers: 0.15, 0.26, 3.15, and 5.79×10^4 neutrophils), which limited the ability to determine significant increases in neutrophils in mice exposed to WTC PM samples in sub-experiment C2. The reasons for this significant inflammatory response in two control mice are not apparent, but this finding does not detract from the overall conclusion that PM collected from specific locations near the WTC site caused significant inflammation of neutrophils and eosinophils.

These results differ substantially from those found in Experiment A, where 100 μg of pooled WTCX induced only a mild neutrophilic response in the lung one day after oropharyngeal aspiration (average 1.43×10^4). Some WTC individual site samples (WTCF, WTC13, WTC11, WTCE) caused about 4 times the amount of neutrophil recruitment as WTCX, while the others (WTC8, WTCB, WTCC) caused about twice as much recruitment. It is not clear how the individual site samples could all cause more lung inflammation than the pooled WTCX sample which was composed of the individual site samples. This finding may be a result of significant differences in responsiveness of different lots of mice sent on different weeks, which we have found with some studies of other toxic inhalants. Additionally, although these mice were lavaged after Mch challenge, our experience has shown that the challenge itself does not induce cellular inflammation that might account for the observations made here. To adequately address this question, pooled WTCX and individual site samples would need to be tested together in the same experiment.

Table 19. Experiment C: BAL Cell Numbers [a]

Group	Sub-Experiment	Day	BAL Cell Number (x 10^{-4}) Mac	Neut	Eos	Lym
Saline	C1	1	**16.41**	**0.34**	**0.02**	**0.14**
			1.92	0.20	0.01	0.02
WTC8	C1	1	**21.43**	**3.23**	**0.27**	**0.38**
			2.38	0.52	0.08	0.05
WTC13	C1	1	**24.19**	**6.10**	**1.33**	**0.70**
			2.24	0.88	0.43	0.14
WTCF	C1	1	**18.47**	**6.85**	**0.84**	**0.58**
			1.45	0.85	0.22	0.10
NIST	C1	1	**17.36**	**14.67**	**0.43**	**0.48**
			3.41	1.17	0.17	0.13
Saline	C1	3	**24.03**	**0.48**	**0.19**	**0.36**
			6.56	0.45	0.07	0.09
WTC8	C1	3	**29.79**	**0.18**	**1.48**	**0.75**
			2.70	0.06	0.90	0.15
WTC13	C1	3	**25.96**	**0.46**	**0.39**	**0.88**
			2.32	0.15	0.10	0.22
WTCF	C1	3	**24.60**	**0.25**	**1.46**	**1.16**
			2.23	0.07	0.49	0.26
NIST	C1	3	**29.09**	**1.74**	**0.67**	**1.97**
			4.06	0.26	0.36	0.41
Saline	C2	1	**16.25**	**2.34**	**0.22**	**0.16**
			1.21	1.34	0.19	0.06
WTC11	C2	1	**23.38**	**5.79**	**0.75**	**0.98**
			3.84	1.42	0.39	0.26
WTCB	C2	1	**25.91**	**2.98**	**0.17**	**0.49**
			3.90	0.95	0.05	0.05
WTCC	C2	1	**27.19**	**2.53**	**0.28**	**0.29**
			4.02	0.42	0.10	0.08
WTCE	C2	1	**24.19**	**5.12**	**0.37**	**0.38**
			2.94	0.80	0.12	0.10
Saline	C2	3	**20.87**	**0.03**	**0.02**	**0.27**
			0.69	0.02	0.01	0.11
WTC11	C2	3	**25.50**	**1.13**	**0.49**	**0.75**
			3.80	0.99	0.16	0.31
WTCB	C2	3	**29.60**	**0.16**	**0.30**	**0.46**
			2.87	0.05	0.12	0.11
WTCC	C2	3	**24.76**	**0.34**	**0.39**	**0.79**
			2.24	0.08	0.14	0.19
WTCE	C2	3	**30.11**	**0.21**	**1.59**	**1.45**
			2.62	0.05	0.43	0.50

[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group, except Saline sub-experiment C2: n=4). Significant differences shown are within sub-experiments only. Heavy solid-line boxes: Within sub-experiment C1 day 1 neutrophils, NIST > WTC13 and WTCF > WTC8 > Saline. Solid-line boxes, underlined values: NIST, WTC13, WTCF, and WTC8 all significantly different from Saline. Dashed-line boxes: WTC11 significantly different from Saline. Solid-line shaded boxes: WTCE significantly different from Saline and WTCB.

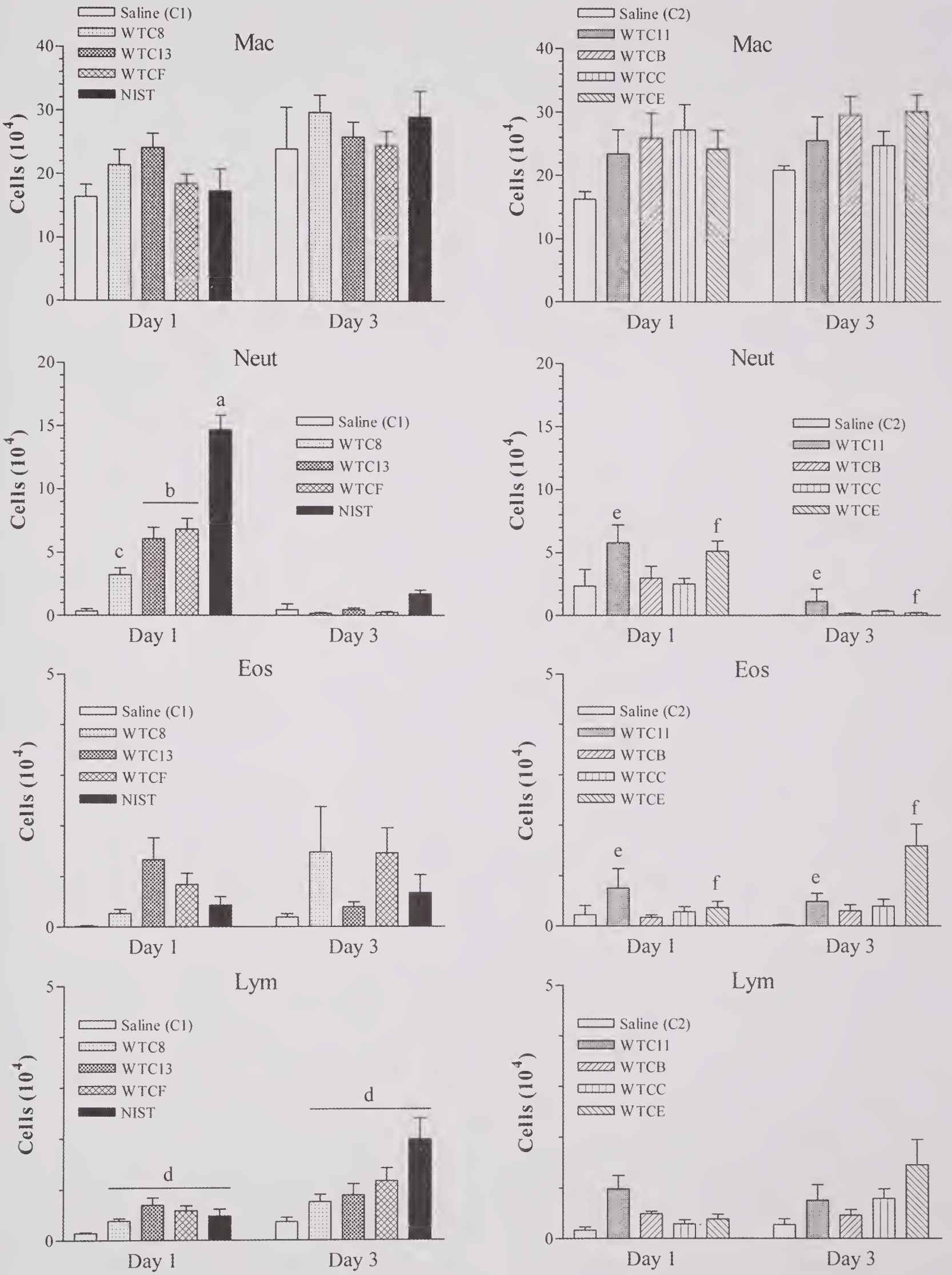


Figure 16. Experiment C: BAL cells. See next page for figure legend.

Figure 16. (previous page.) Experiment C: BAL cell numbers recovered from mice exposed to saline vehicle, NIST 1649a, or WTC PM samples from individual collection sites and tested 1 or 3 days later (n = 8 per group except Saline sub-experiment C2: n = 4). Cell types shown are macrophages (Mac), neutrophils (Neut), eosinophils (Eos), and lymphocytes (Lym). [a] NIST significantly greater than all other groups. [b] WTC13 and WTCF significantly greater than WTC8 and Saline C1 groups. [c] WTC8 significantly greater than Saline C1 group. [d] Lymphocyte numbers significantly greater in WTC8, WTC13, WTCF, and NIST groups compared with Saline C1 group. [e] Significantly greater numbers of neutrophils and eosinophils in WTC11 group compared with Saline C2 group. [f] Significantly greater numbers of neutrophils and eosinophils in WTCE group vs. WTCB and Saline C2 groups.

4. BAL proteins and enzymes. As for other parameters, BAL protein and enzyme data for sub-experiments C1 and C2 were analyzed separately (Table 20, Figure 17). In sub-experiment C1, significant increases in BAL total protein levels were found in the NIST group compared with the WTC8 group ($P = 0.05$). No significant differences due to Treatment were found with respect to albumin or LDH levels. There were significant interactions between Day and Treatment in NAG values ($P = 0.02$), indicating the results depended on the day animals were killed.

In sub-experiment C2, there were significant effects of Day after treatment for total protein and LDH, but there were no effects of Treatment group. There were significant interactions between Day and Treatment in NAG values ($P = 0.005$), indicating the results depended on the day after treatment.

In both sub-experiment C1 and C2, one of the saline group mice killed on Day 1 had very high values for total protein, albumin, and LDH, which increased the mean values and variability in these groups. Although this result may have limited the ability to detect some statistical differences, overall the biochemical values were not greatly different among the treatment groups, and any additional differences with more consistent control data would likely have been minimal. Therefore the results for the individual site WTC PM samples are comparable to those found with the pooled WTCX sample in Experiment A, where no differences from control saline mice were found.

5. Lung histopathology. Following tests for airway responsiveness to Mch aerosol and lung lavage, lungs were removed and fixed with 4% paraformaldehyde, and pathological changes were assessed. Although the lungs of all mice in Experiment C were lavaged (they were not lavaged in Experiments A or B), the pattern and the morphology of the PM induced findings were relatively consistent in all treated groups.

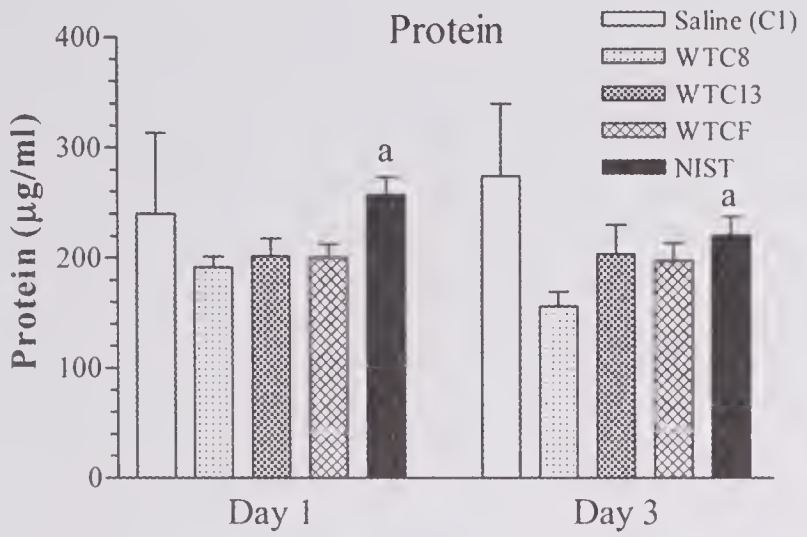
Focal subacute bronchiolar inflammation and focal bronchiolar pigmented macrophages (presumably PM) were consistently observed in all groups of mice dosed with each of the different PM samples, and both findings are considered to be PM-induced in all groups (Table 21). Some groups also had findings of focal free bronchiolar pigment, consistent with the pigment in macrophages. No remarkable findings were observed in the lungs of the saline control group (Figure 18A), except for one mouse which had a minimal degree of focal subacute bronchiolar inflammation which was not considered to be treatment-related. Table 21 shows the rankings of the treatment-related histopathologic findings in mice 1 or 3 days after exposure. The degree of focal subacute bronchiolar inflammation was greatest in the NIST (Figure 18C), WTCE, and WTC13 (Figure 18D) groups on Day 1 (average severity scores of 1.9, 2.0, and 2.1, respectively). The scores in the WTCC (Figure 18B), WTCB, WTC8, WTCF, and WTC11 groups were lower (average severity scores of 0.8, 1.1, 1.1, 1.3, and 1.3, respectively). By Day 3, the focal subacute bronchiolar inflammation was greatest in the NIST group (average severity score 2.1; Figure 18E), while the scores were reduced in all of the WTC PM groups relative to their scores on Day 1 (Figure 18F).

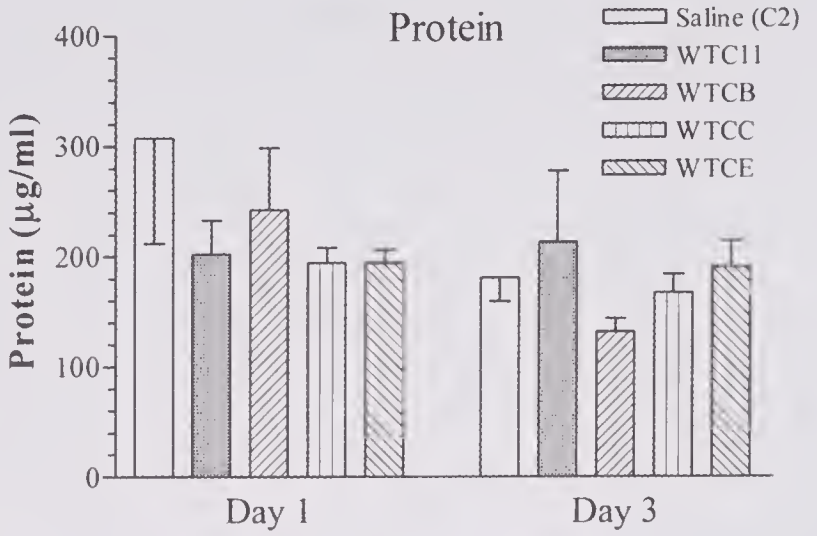
The histopathologic scoring system is semi-quantitative, and much larger numbers of mice per group would be necessary to determine statistically significant differences among groups. Nevertheless, these results also show substantial differences from those found in Experiment A with the pooled WTCX sample. Oropharyngeal aspiration of 100 μg of WTCX did not cause any treatment-related histopathologic findings. In contrast, all individual site samples of WTC PM induced at least minimal focal subacute bronchiolar inflammation, and some samples caused slight/mild and even moderate degrees of inflammation. In addition, pigment associated with PM was visible in macrophages from all WTC PM-exposed mice, but none was visible in mice exposed to pooled WTCX in Experiment A. Re-examination of the slides by a different observer will be necessary to confirm this finding. The findings of pulmonary inflammation in WTC PM groups by histopathologic examination are consistent with the results from the quantification of BAL cell numbers.

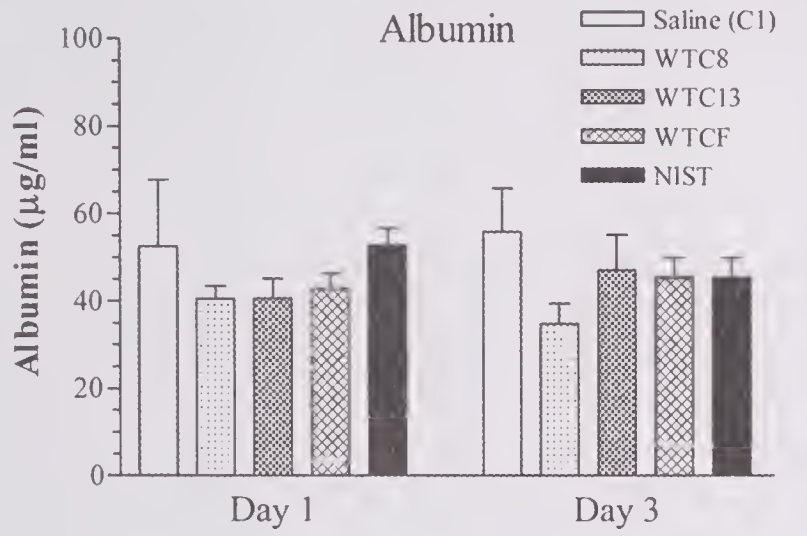
Table 20. Experiment C: BAL Supernatant Biochemical Values [a]

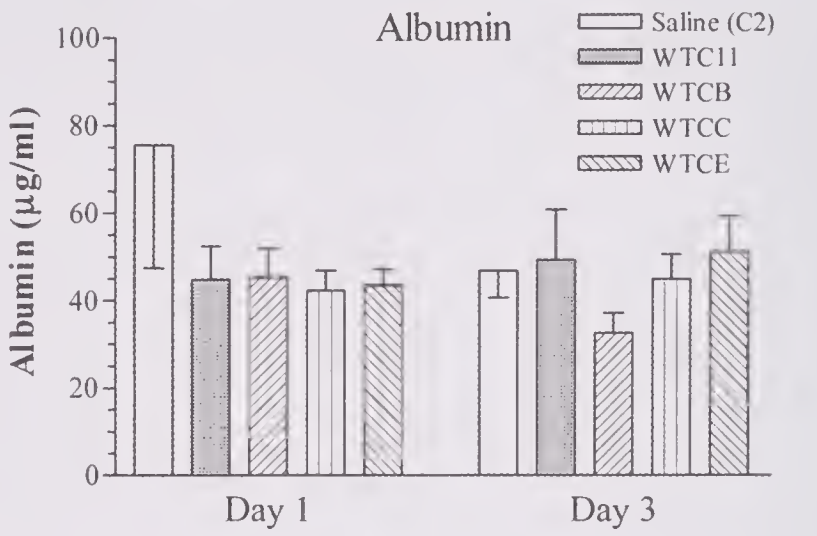
Group	Sub-Experiment	Day	Protein µg/ml	Albumin µg/ml	LDH U/L	NAG U/L
Saline	C1	1	**240.0**	**52.5**	**43.1**	**1.7**
			73.9	15.2	5.3	0.2
WTC8	C1	1	**191.9**	**40.5**	**46.1**	**2.7**
			9.5	3.1	3.6	0.3
WTC13	C1	1	**201.8**	**40.6**	**40.6**	**2.9**
			16.3	4.5	4.4	0.2
WTCF	C1	1	**200.4**	**42.9**	**44.6**	**1.7**
			11.9	3.4	3.3	0.2
NIST	C1	1	**257.1**	**52.6**	**56.8**	**4.5**
			16.3	4.1	5.0	0.4
Saline	C1	3	**274.6**	**55.9**	**37.5**	**3.1**
			65.4	10.0	11.1	1.3
WTC8	C1	3	**156.1**	**34.9**	**35.2**	**2.3**
			13.3	4.6	1.8	0.1
WTC13	C1	3	**203.8**	**47.1**	**36.0**	**2.7**
			26.4	8.1	2.2	0.2
WTCF	C1	3	**197.5**	**45.4**	**37.2**	**2.9**
			15.9	4.5	2.8	0.4
NIST	C1	3	**220.4**	**45.2**	**40.2**	**4.3**
			17.3	4.6	5.2	0.6
Saline	C2	1	**307.8**	**75.5**	**44.5**	**2.9**
			95.7	28.0	18.9	0.2
WTC11	C2	1	**202.0**	**44.8**	**48.1**	**2.4**
			30.8	7.7	6.2	0.7
WTCB	C2	1	**242.5**	**45.2**	**55.2**	**2.9**
			56.4	6.6	13.0	0.8
WTCC	C2	1	**194.8**	**42.2**	**34.6**	**1.7**
			13.5	4.6	2.6	0.1
WTCE	C2	1	**194.5**	**43.5**	**39.5**	**3.0**
			11.7	3.6	2.7	0.4
Saline	C2	3	**180.8**	**46.8**	**29.5**	**2.3**
			21.1	6.1	4.9	0.3
WTC11	C2	3	**213.0**	**49.2**	**50.1**	**4.4**
			64.7	11.5	15.0	2.3
WTCB	C2	3	**131.8**	**32.5**	**32.4**	**2.2**
			12.2	4.6	3.6	0.1
WTCC	C2	3	**167.2**	**44.8**	**30.0**	**2.9**
			16.4	5.8	1.7	0.2
WTCE	C2	3	**190.2**	**51.0**	**29.4**	**2.5**
			23.4	8.1	2.1	0.2

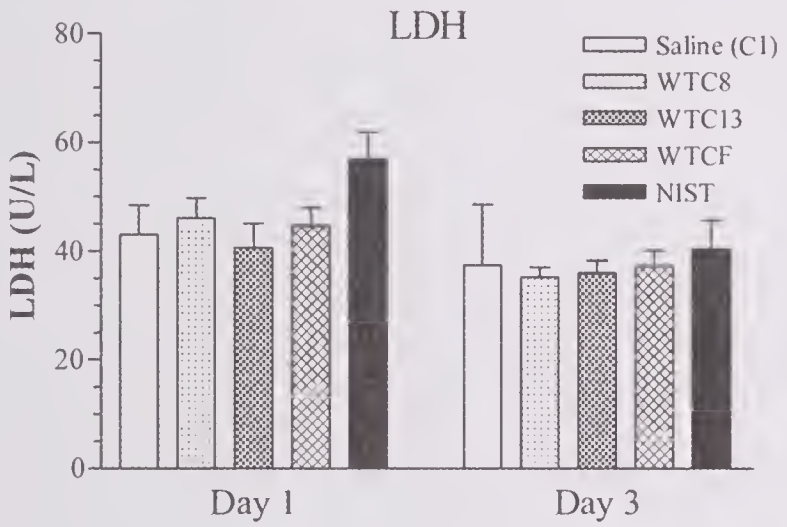
[a] Values shown are means (in bold) and SEM immediately below means (n=8 per group, except Saline sub-experiment C2: n=4). Significant differences shown are within sub-experiments only. Heavy solid-line boxes: NIST significantly different from WTC8. Significant overall Day effects were found for Protein (sub-experiment C2), and LDH (both sub-experiments).

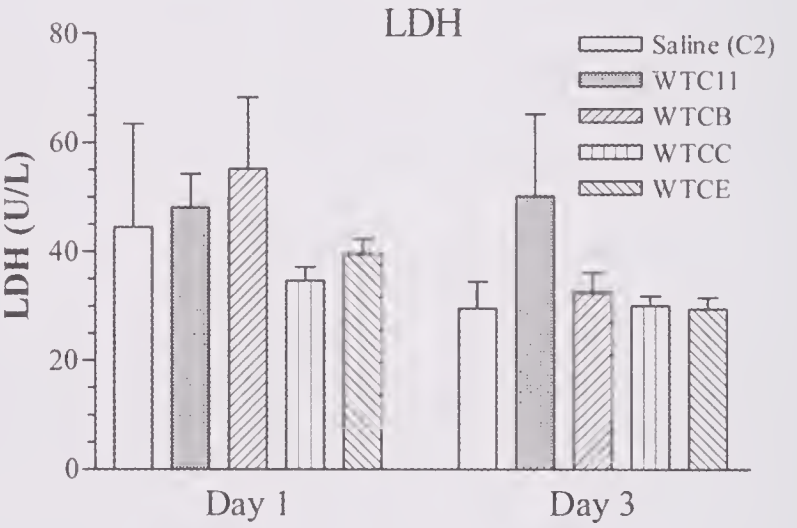


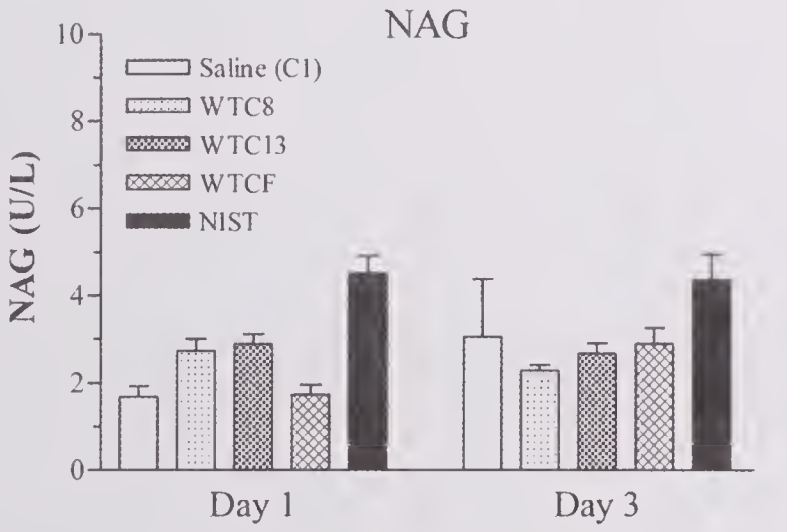












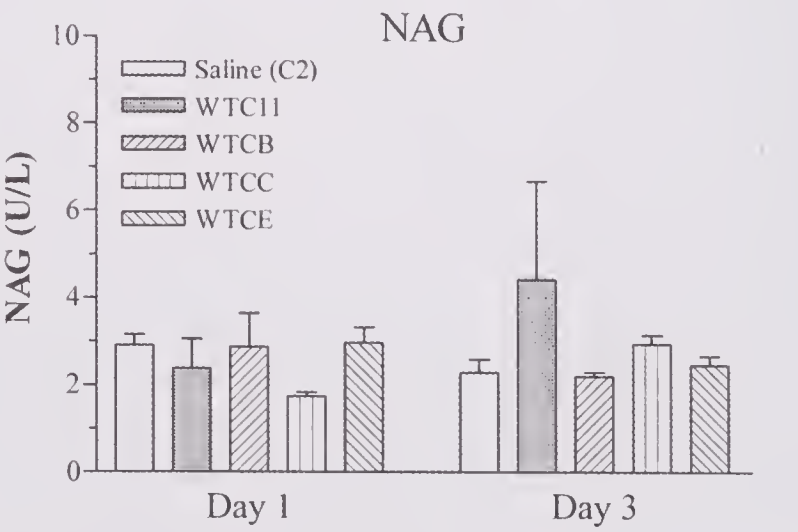


Figure 17. Experiment C: BAL supernatant biochemistry. See next page for figure legend.

Figure 17. (previous page.) Experiment C: Bronchoalveolar lavage supernatant proteins and enzymes recovered from mice exposed to saline vehicle, NIST 1649a, or WTC PM samples from individual collection sites and tested 1 or 3 days later (n = 8 per group except Saline sub-experiment C2: n = 4). [a] Significantly greater protein values in NIST group vs. WTC8 group. Other significant differences were found due to Day of sacrifice or interactions between Day and Treatment, but there were no other effects due to Treatment alone.

*6. **Summary.*** Examination of the effects of WTC PM collected from different locations surrounding the WTC site showed that all samples were capable of inducing pulmonary inflammation and hyperresponsiveness to Mch aerosol, although overt lung damage as determined by biochemical parameters of lung injury was minimal. The neutrophilic response was substantially greater for all individual site WTC PM samples compared with the response induced by the pooled WTCX sample in Experiment A, although differing responsiveness of different shipments of mice could account for this finding, and a direct comparison would be necessary to determine if there is a difference. Numbers of neutrophils declined from Day 1 to Day 3 after oropharyngeal aspiration, as determined by both BAL and histopathologic examination. Other cell types appeared to be more persistent or increase from Day 1 to Day 3 (especially lymphocytes in sub-experiment C1), but these were not large changes. Respiratory responsiveness to Mch aerosol was significantly increased in all WTC groups compared with saline controls, although mice exposed to WTC13 were less responsive than other WTC groups. The degree of Mch hyperresponsiveness in the WTC groups of Experiment C appeared to be comparable to that from the WTCX group in Experiment A.

No particular geographical significance could be deduced from the patterns of responses induced by the individual WTC PM samples. The one group which had

Table 21. Experiment C - Summary of Treatment-Related Histopathologic Findings in Mice 1 or 3 Days after Intratracheal Instillation of Particulate Matter Samples [a]

Treatment Group	Sub-Experiment	Day	Bronchiole, Inflammation, Subacute, Focal		Bronchiole, Pigment, Macrophage, Focal		Bronchiole, Pigment, Free, Focal	
			Incidence	Severity	Incidence	Severity	Incidence	Severity
WTC13	C1	1	8/8	2.1	8/8	2.0	4/8	0.6
WTCE	C2	1	8/8	2.0	8/8	1.9	2/8	0.3
NIST	C1	1	8/8	1.9	7/8	2.0	7/8	0.9
WTC11	C2	1	8/8	1.3	7/8	0.9	0/8	0.0
WTCF	C1	1	8/8	1.3	6/8	0.8	0/8	0.0
WTC8	C1	1	6/8	1.1	6/8	0.8	0/8	0.0
WTCB	C2	1	6/8	1.1	6/8	0.8	0/8	0.0
WTCC	C2	1	6/8	0.8	4/8	0.5	0/8	0.0
Saline	C1	1	1/8	0.1	0/8	0.0	0/8	0.0
NIST	C1	3	8/8	2.1	8/8	2.0	0/8	0.0
WTC11	C2	3	6/8	1.1	2/8	0.3	0/8	0.0
WTCE	C2	3	6/8	0.8	6/8	0.8	0/8	0.0
WTC8	C1	3	4/8	0.8	1/8	0.1	0/8	0.0
WTC13	C1	3	4/8	0.6	3/8	0.4	0/8	0.0
WTCB	C2	3	3/8	0.4	2/8	0.3	0/8	0.0
WTCF	C1	3	3/8	0.4	1/8	0.1	0/8	0.0
WTCC	C2	3	2/8	0.3	1/8	0.1	0/8	0.0
Saline	C1	3	0/7	0.0	0/7	0.0	0/7	0.0

[a] Saline-instilled control mice in sub-experiment C2 were not examined. Incidence denotes number of mice in group with finding / total number of mice examined. Average severity score for the group is shown based on the following scoring system: 0 = not present, 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high. Groups are arranged in descending order of severity within each post-exposure day, first by severity of focal subacute bronchiolar inflammation, and then by severity of focal bronchiolar pigmented macrophages.

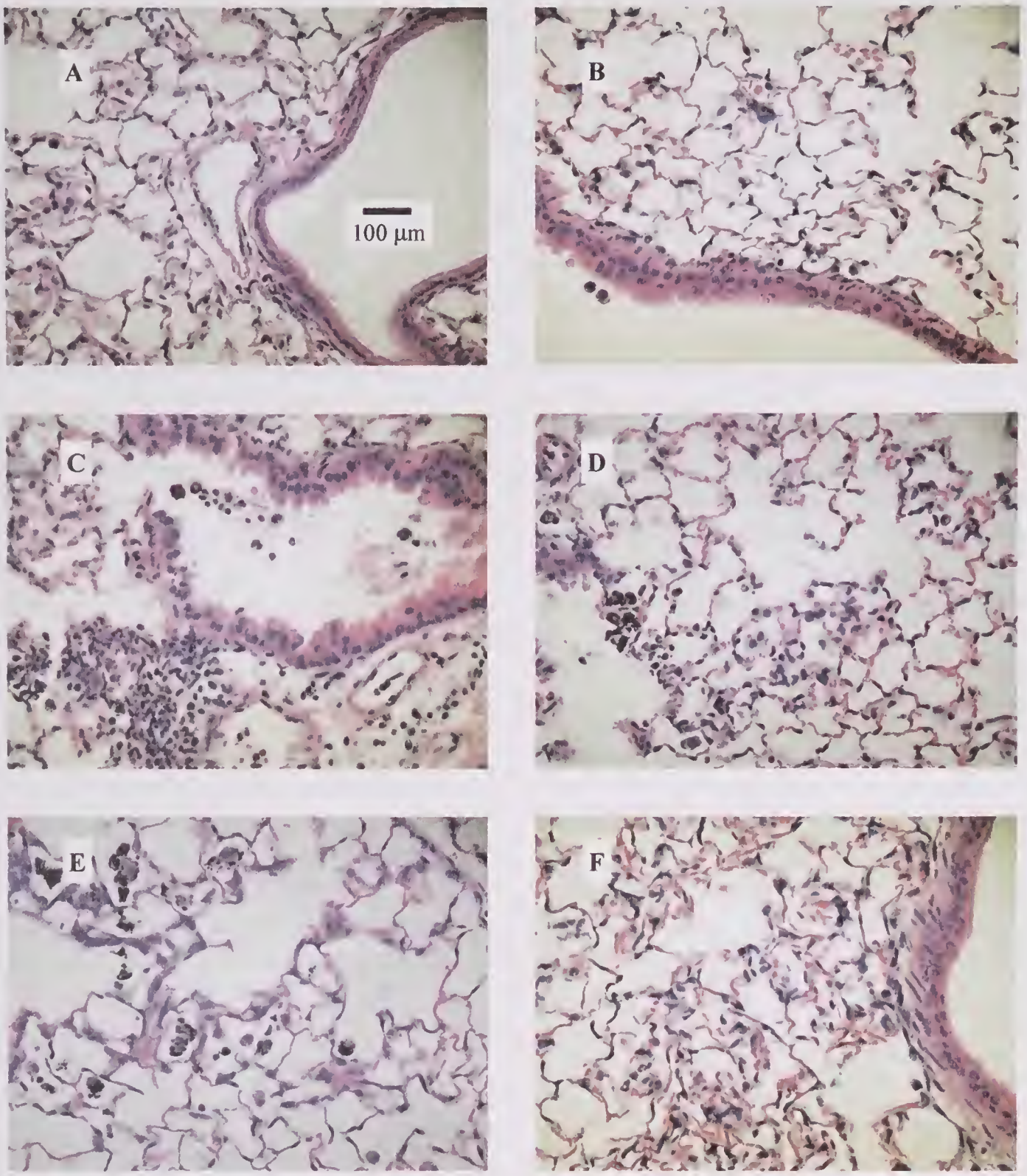


Figure 18. Experiment C. Representative micrographs of lesions occuring in lungs of mice 1 or 3 days after intratracheal instillation of 100 µg PM sample or saline vehicle (all panels same magnification: bar length = 100 µm). A. Saline-instilled control mouse (#301), Day 1, with no remarkable findings. B. Mouse #349 instilled with WTCC, Day1, showing minimal degree of focal subacute bronchiolar inflammation (FSBI). C. Mouse #291 instilled with NIST, Day 1, with moderate degree of FSBI. D. Mouse #270 instilled with WTC13, Day 1, with moderate degree of FSBI. E. Mouse #300 instilled with NIST, Day 3, with slight/mild degree of FSBI. F. Mouse #280 instilled with WTC13, Day 3, with minimal degree of FSBI.

lower Mch responsiveness (WTC13) was centrally located only 0.1 mile southeast of the center of Ground Zero. The WTCF sample was blown into a building at 120 Broadway and collected on an undisturbed marble staircase. The responses caused by this "indoor" sample were quite similar to those caused by the other "outdoor" WTC PM samples.

In general, responsiveness to Mch aerosol and pulmonary inflammation were not well correlated. Mice in the WTC13 group had one of the largest neutrophilic and eosinophilic responses, yet had a significantly lower degree of Mch responsiveness. Mice in the WTCC group had perhaps the greatest response to Mch challenge (not significantly different from WTCB, WTCE, or WTC11),

yet their neutrophil and eosinophil responses were low relative to the other WTC groups. As noted previously, a lack of correlation between inflammation and airway hyperresponsiveness is not uncommon (Alvarez et al., 2000; Smith and McFadden Jr., 1995).

IV. Discussion

Samples of fallen dust were collected at various locations in the immediate vicinity of the WTC site one and two days after the WTC disaster, and were examined by several physical and chemical techniques. Both coarse unfractionated and fine size-fractionated WTC PM samples were composed primarily of calcium-based compounds such as calcium sulfate (gypsum) and calcium carbonate (calcite; the main component of limestone). These and other compounds and elements found in the WTC PM samples are indicative of crustal material-derived building materials such as cement, concrete aggregate, ceiling tiles, and wallboard. Both gypsum and calcite irritate the mucus membranes of the eyes, nose, throat, and upper airways (Stellman, 1998). Calcium carbonate dust causes coughing, sneezing, and nasal irritation (NLM, 2002). These minerals are often contaminated with small amounts of silica, which is the main concern for occupational health hazards (Stellman, 1998). Minor amounts of silica (quartz) were detected in the WTC PM samples

Our chemical analysis generally agrees with the extensive analysis of WTC PM performed by the USGS (USGS, 2002). Levels of carbon were relatively low, suggesting that combustion-derived particles did not form a significant fraction of these samples recovered in the immediate aftermath of the destruction of the towers. Lastly, there was no evidence of significant asbestos contamination of the samples used in these studies, although the physical analyses conducted were not specifically focused on definitive asbestos quantitation. As of May 23, 2002, the U.S. EPA had analyzed 9,544 air samples in Lower Manhattan since September 11, and found elevated levels of asbestos in only 21 samples (EPA, 2002c).

The effects of exposure to samples of WTC $PM_{2.5}$ on respiratory parameters, pulmonary inflammation, and lung injury were investigated in young adult female CD-1 mice, an outbred strain expected to have significant variability in biological responses, in three separate experiments. A pooled sample of WTC $PM_{2.5}$ composed of roughly equivalent amounts of samples from 7 different locations around the WTC site caused a mild degree of pulmonary inflammation in mice (7% neutrophils in BAL fluid), and had no effect on parameters of acute lung injury at a dose of 100 μg instilled directly into the lungs. ROFA, a toxic positive control fine PM sample, caused a much higher degree of lung inflammation and lung injury at the same dose. However, mice instilled with 100 μg pooled WTC $PM_{2.5}$ had highly significant increases in airway responsiveness to methacholine (Mch) aerosol challenge, which were significantly greater than that of ROFA. Mice exposed to lower doses of pooled WTC $PM_{2.5}$ (10 μg and 31.6 μg) and mice exposed by nose-only inhalation (estimated to have about 14 μg WTC $PM_{2.5}$ deposited in the respiratory tract) did not have any biologically significant changes in methacholine responsiveness or neutrophilic inflammation. These dose-response relationships and the lack of effect in nose-only exposure suggest that inhalation of relatively high doses of WTC $PM_{2.5}$ are necessary to elicit respiratory effects in people.

Mice exposed to samples of WTC $PM_{2.5}$ from the 7 individual sites around Ground Zero had greater lung inflammation (2 to 4-fold) than mice exposed to the WTC $PM_{2.5}$ sample pooled from these sites. These findings occurred in separate experiments and would need to be confirmed by a direct comparison, but nonetheless all groups of mice exposed to the individual site samples developed hyperresponsiveness to Mch aerosol challenge, similar to mice exposed to the pooled sample. No particular pattern of responses was found corresponding to the geographical location where the samples were taken. Pulmonary inflammation in mice exposed to individual site WTC $PM_{2.5}$ samples diminished from 1 day to 3 days after exposure, although hyperresponsiveness to Mch aerosol did not diminish significantly. Further experiments would be necessary to determine the persistence of pulmonary responses in mice, which may lead to insights into whether any WTC PM-associated effects which may exist in people are persistent.

The results of these studies should be examined in the context of previous studies of the effects of

environmentally relevant PM samples in rodents. Rats were intratracheally instilled with 2.5 mg (~8.3 mg/kg) of various emission source and urban ambient air PM samples (Costa and Dreher, 1997), a dose about twice as high, based on body weight, as the 100 μg WTC $PM_{2.5}$ dose in mice (~4 mg/kg). Oil fly ashes and urban ambient air PM samples (including a ROFA similar to the one used in the present study and NIST1649a) induced strong neutrophilic responses 24 hr after exposure, while biochemical markers of lung injury were lower in the urban air PM samples compared with the oil fly ash samples. ROFA at this dose induced airway hyperresponsiveness in rats which persisted at least 4 days, and was greater than that observed in an urban ambient air PM sample (Pritchard et al., 1996). The fact that WTC $PM_{2.5}$ induced a significantly greater degree of airway hyperresponsiveness in mice than ROFA, which is used as a toxic positive control particle in many studies, suggests a very significant respiratory effect of a relatively high dose exposure to WTC $PM_{2.5}$.

Some people were exposed acutely to high concentrations of dust in the WTC disaster, and subsequently developed wheezing or symptoms of sensory irritation, such as cough and irritation of the nose and throat. These effects resemble, in some respects, the reactive airways dysfunction syndrome (RADS). RADS can occur after single or multiple high-level occupational exposures to an irritating vapor, fume, or smoke (Gautrin et al., 1999). Effects can occur within minutes or hours after exposure, and include cough, dyspnea, and wheezing. Clinical tests can show airways obstruction, persistent airway hyperresponsiveness, and inflammation. The recovery process appears to be dependent on the initial degree of injury. The effects of a high dose exposure to WTC $PM_{2.5}$ in mice (100 μg) appear to mimic at least some of these responses, especially the significant increase in airway hyperresponsiveness to Mch. It is important to note that WTC $PM_{2.5}$-induced pulmonary inflammation, although significantly greater than in control mice, was not as robust as one might expect in a realistic animal model of RADS. However, the degree to which inflammation and airway hyperresponsiveness are associated in RADS is not clear (Gautrin et al., 1999). Examination of other time points would be necessary to determine the persistence of WTC PM-induced airway hyperresponsiveness in mice and its similarity to RADS.

Close examination of the data suggested that individual mice within the outbred CD-1 strain vary in sensitivity to the effects of WTC $PM_{2.5}$. Certain individuals within the human population may also have particular susceptibility to the hazards posed by exposure to WTC $PM_{2.5}$. It is known that some asthmatic individuals are hyperresponsive to nonspecific irritants such as cold dry air (Anderson and Daviskas, 2000) or cigarette smoke (Bonham et al., 2001). This subpopulation is likely to be at high risk for development of dust-induced airways obstruction (Donaldson et al., 2000; Peden, 2001; Nel et al., 2001). Very few studies have been published regarding the effects of alkaline aerosols on pulmonary function in asthma. One study reported that inhalation of high concentrations of an alkaline aerosol (pH 9.8 to 10.3) had no significant effect on irritant symptoms or specific airways resistance in mild asthmatic patients (Eschenbacher, 1991). However, this aerosol was composed of a simple mixture of sodium carbonate, sodium bicarbonate, and sodium hydroxide. The chemical composition of the alkaline (pH 8.88 to 10.00) WTC $PM_{2.5}$ is much more complex and interactions of numerous chemical species may be associated with development of airway hyperresponsiveness to methacholine or other bronchoconstrictors.

How does the dose of 100 μg WTC $PM_{2.5}$, which caused bronchiolar inflammation and airway hyperresponsiveness in mice, relate to exposure of people at the WTC site? Because inflammation was observed mainly in the airways, and airway hyperresponsiveness is mainly due to dysfunction of airway smooth muscle (Fredberg, 2000), the dose metric which is probably most relevant is dose per surface area of the tracheobronchial (TB) region of the respiratory tract. The TB region is defined as the airways (excluding the nasal (head) region) from the trachea down to the terminal bronchioles (Overton et al., 2001). Therefore, to assess the risks of exposure in people, the concentrations of WTC $PM_{2.5}$ in air which could produce doses per TB surface area in humans equivalent to that in mice should be calculated. These WTC $PM_{2.5}$ concentrations may be estimated (Table 22) using the following assumptions: 1) The mouse alveolar pulmonary surface area can be estimated from an allometric equation based on body weight (Jones and Longworth, 1992), and the TB surface area is very small in comparison to the alveolar surface area (Overton et al., 2001); 2) Oropharyngeal aspiration bypasses the mouse nose and spreads the dose of WTC $PM_{2.5}$ evenly over the TB and pulmonary alveolar surface areas of the mouse lung; 3) The human TB dose per surface area, selected to match the mouse dose per surface area, does not clear from the lung in the time frame of exposure to WTC $PM_{2.5}$ (an 8-hour work shift was selected); and 4) The model of the fraction of inhaled $PM_{2.5}$ (model particles with MMAD = 1, σg = 2.5, and density = 1 g/cc) deposited in the TB region (Freijer et al., 1999) assumes a reference 30 year-

Table 22 Estimation of WTC $PM_{2.5}$ Concentrations Required to Produce Human Doses Equivalent to Mouse Doses Used in WTC2001 Study

Dose deposited in mouse tracheobronchial and pulmonary regions (μg)	10	31.6	100
Mouse alveolar pulmonary surface area (m^2) [a]	0.103	0.103	0.103
Mouse dose per tracheobronchial (TB) or pulmonary surface area (mg/m^2) [b]	0.097	0.307	0.973
Human TB surface area (m^2) [c]	0.415	0.415	0.415
Total human TB dose equivalent to mouse TB dose ($mg/m^2 \times m^2$) [d]	0.040	0.128	0.404
Deposition fraction in human TB region [e]	0.066	0.066	0.066
Total inhaled dose in mg (total human TB dose / TB deposition fraction)	0.612	1.932	6.115
Quantity of air breathed in 8 hr workshift at ventilation of 30 L/min (m^3) [f]	14.4	14.4	14.4
WTC $PM_{2.5}$ concentrations required to produce human doses equivalent to mouse doses used in WTC2001 Study ($\mu g/m^3$)	42	134	425

[a] From Jones and Longworth (1992) calculated allometric equation: Mammalian alveolar pulmonary surface area in $m^2 = 3.36 \times (Wt, kg)^{0.935}$, where weight = 0.024 kg (average mouse weight in all studies). Tracheobronchial surface area is minimal in comparison to alveolar surface area and can be ignored in calculation.

[b] Assumes dose is spread out evenly over tracheobronchial and pulmonary alveolar regions.

[c] Based on 30 year old, 5' 10" male with functional residual capacity (FRC) of 3300 ml (Overton et al., 2001).

[d] Calculations assume no clearance of particles after deposition in human respiratory tract.

[e] Calculations made with Multiple Path Particle Deposition model version 1.11 (Freijer et al., 1999) which assume human Yeh-Schum 5-lobe model, FRC = 3300 ml (appropriate for 30 year old, 5'10" male), upper respiratory tract volume = 50 ml, density of particles = 1 gm / cc, diameter = 1 μm MMAD, inhalability adjustment on, $\sigma g = 2.5$, breathing frequency = 15 min^{-1}, tidal volume = 2000 ml, minute volume = 30 L/min, inspiratory:expiratory ratio = 1, and oronasal mouth breathing.

[f] Estimate of minute ventilation during moderate to heavy sustained work (Åstrand and Rodahl, 1986).

old 5' 10" male breathing oronasally with a minute ventilation of 30 L/min (estimate during moderate to heavy sustained work; Åstrand and Rodahl, 1986). The total human TB dose and the fraction deposited in the TB region are used to back-calculate the total inhaled dose of $PM_{2.5}$. The total inhaled dose divided by the quantity of air breathed in a typical 8-hour work shift yields the concentration of $PM_{2.5}$ in the WTC work or neighborhood environment required to produce human doses equivalent to the mouse doses used in the WTC2001 study (Table 22). These calculations show that under these conditions, concentrations of 42, 134, and 425 $\mu g/m^3$ WTC $PM_{2.5}$ would produce human doses per TB surface area equivalent to the mouse doses of 10, 31.6, and 100 μg, respectively. Obviously many factors may cause wide variations in the calculation of dose, and extrapolation of responses from the mouse to the human involves another dimension of uncertainty which was not considered, but it seems reasonable to say that a healthy worker breathing heavily in the dusty environment generated after the collapse of the towers could have inhaled enough $PM_{2.5}$ to approximate the 100 μg dose in the mouse. Therefore, inhalation of a very high concentration of WTC $PM_{2.5}$ (e.g. ~425 $\mu g/m^3$) over a short period of time (8 hr) could have contributed to development of pulmonary inflammation, airway hyperresponsiveness, and manifestations of sensory irritation such as cough. Individuals who are especially sensitive to inhalation of dusts, such as asthmatics, may experience these effects at lower doses of inhaled WTC $PM_{2.5}$. However, most healthy people would not be expected to respond to moderately high WTC $PM_{2.5}$ levels (130 $\mu g/m^3$ or less for 8 hours) with any adverse respiratory responses. The effects of chronic or repeated exposures to lower levels of WTC $PM_{2.5}$, or the persistence of any respiratory effects are unknown and were not components of this study. The persistence of any effects of inhaled WTC $PM_{2.5}$, if similar to RADS, would be expected to depend on the dose initially deposited in the respiratory tract.

It is important to consider several limitations of these studies. First, most of the experiments used oropharyngeal aspiration to deliver PM samples to the respiratory tract

rather than more physiologically relevant inhalation exposure methodology. We believe that utilizing oropharyngeal aspiration, as described in the Experimental Design section, had many advantages and was necessary in these circumstances. However, this report indicates that future studies may be needed to more closely examine bronchoconstriction and sensory irritation during inhalation exposure to WTC PM in mice and in guinea pigs, a species known to be especially sensitive to sensory irritants (Costa and Schelegle, 1999). Secondly, these studies only evaluated short-term toxicological effects (endpoints were examined 1, 3, or 6 days after exposure) after acute exposure and no direct information is provided on the long term effects of acute or chronic exposures to WTC $PM_{2.5}$. Thirdly, gaseous and vapor-phase toxicants (e.g. dioxin and volatile organic compounds such as benzene) were certainly released, especially during the fires which continued for months after September 11 (EPA, 2002c). The collection and processing techniques described in this report do not allow investigation of these important toxic species, nor are the interactions of particles with gases or organic vapors considered (Mautz et al., 2001). Finally, these studies only examined fine $PM_{2.5}$, while the toxicity of coarse mode and larger size PM fractions were not investigated. However, it is important to remember that the size-fractionation techniques employed in this report are not absolute, and significant quantities of PM > 2.5 μm are present in the samples. Furthermore, analysis of the WTC $PM_{2.5}$ and PM_{53} samples showed that they were similar in composition (Tables 3 and 5), suggesting that only differences in respiratory tract deposition patterns of fine and coarse WTC PM would affect biological responses. Coarse mode PM may be more relevant for upper airways sensory irritation because larger particles will mainly deposit in the upper airways where sensory innervations are predominant (Costa and Schelegle, 1999). However, chronic effects of fine PM may be greater than coarse PM since it can be inhaled more deeply and deposit in peripheral regions of the lungs, and is more slowly cleared. Coarse PM is much less inhalable in small rodents than in humans, and less is deposited in the respiratory tract (Menache et al., 1995). Consequently, interpretation of results derived from exposure of mice to coarse PM is problematic, and small rodents are probably not the ideal species to study effects of coarse PM. Nevertheless, because upper airways irritant responses seem to be so important in people exposed to WTC-derived dust, future studies should examine the specific toxicity of coarse WTC PM on respiratory responses in appropriate animal models.

V. Quality Assurance Statement

U.S. EPA World Trade Center Research Project:

"Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center"

The study "Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center" was conducted by the Pulmonary Toxicology Branch, Experimental Toxicology Division (ETD), National Health and Environmental Effects Research Laboratory (NHEERL), Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, in compliance with NHEERL QA Guidelines. Results of these inspections were reported directly to the Principal Investigator (PI) of the Study, Dr. Stephen Gavett. Critical phases in the study were audited.

Date of Inspection	Item Inspected
October 29, 2001	Particulate Matter (PM) filters delivered to EPA.
October 31, 2001	Attempted scraping of PM from filters.
November 2, 2001	Approval of study protocol.
November 2, 2001	Extraction of PM from filters.
November 8, 2001	Shipment of WTC dust samples for endotoxin testing.
November 5-14, 2001	Conduct of Experiment A1-A3: weighing of samples and mice, randomization of mice, dosing of mice, BUXCO, DLCO, BAL, cell counts, methacholine responses, lung samples.
November 15, 2001	Delivery of NIST samples and blank filters.
November 19, 2001	Delivery of #3 Cortland Sample. Shipment of NIST samples for endotoxin testing.
November 26, 2001	Delivery of Experiment B inhalation sample (WTC 3).
November 27, 2001	Conduct of Experiment B (Day 0): placing and removal of mice in inhalation chambers, operation of inhalation pump. Shipment of #3 Cortland Sample Back to NYU.
December 3, 2001	Completion of Experiment B (Day 6): Nasal fixation. Receipt of endotoxin results on WTC dust samples.

Date of Inspection	Item Inspected
December 4, 2001	XRF/XRD laboratory tour. Shipment of 96 mice heads to Michigan State University.
December 11, 2001	Conduct of Experiment C (Day 0): Dosing of Mice. XRF/XRD technical meeting. Receipt of NIST endotoxin test results.
December 27, 2001	Shipment of 184 lung tissues for histopathological analysis.
January 15-17, 2002	Technical Systems Review of project. Interviews with study personnel and inspection of project data and records.
January 16, 2002	Shipment of six PM samples, and 12 PM samples and 10 filter samples for chemical analysis.
January 16-25, 2002	Data audit of spreadsheets against notebooks.
January 30, 2002	Shipment of 10 liquid samples for chemical analysis.
March 15, 2002	Transfer of custody of 12 PM Samples from the EPA Chemist to the PI.
March 4-19, 2002	Data audit of Draft Final Report.

The Quality Assurance Manager of ETD and the Director of Quality Assurance for NHEERL have determined by the above review process that the conduct of this project was in compliance with EPA quality requirements and the operating procedures and study protocol (Intramural Research Protocol No.: IRP-NHEERL-H/ETD/PTB/SHG/01-01-000). Furthermore, the results accurately reflect the raw data obtained during the course of the study.

/ s / 03/22/2002

Thomas J. Hughes, ETD QA Manager Date

/ s / 03/22/2002

Brenda T. Culpepper, NHEERL Director of QA Date

VI. References

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